Applications of Enzymatic Methods to the Preparation of Enantiomerically Pure Compounds

by

Ian Harvey

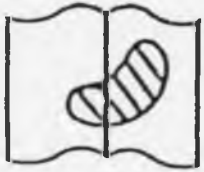
Submitted for the degree of Doctor of Philosophy

University of Warwick

Department of Chemistry

December 1989

BEST COPY
AVAILABLE



Contents

List of Tables and Figures

Abbreviations

Me	methyl
Et	ethyl
Ph	phenyl
Ac	acetate
Pr	propionate
Bu	butyrate
i-Bu	isobutyrate
Va	valerate
St	stearate
R	alkyl or aryl
Tos	toluene-4-sulphonyl

NMR	Nuclear magnetic resonance
s	singlet
d	doublet
t	triplet
q	quartet
m	multiplet
br	broad
J	coupling constant
ppm	parts per million
TMS	tetramethylsilane
csr	chiral solvating reagent

IR	Infra - red
s	strong
m	medium
w	weak
UV	Ultra - violet
MS	Mass spectrum
CI	chemical ionisation
EI	electron impact
c.d.	circular dichroism
[α]	Specific rotation
c	concentration (g/100 ml)
ee	enantiomeric excess
glc	Gas - liquid chromatography
hplc	High pressure liquid chromatography
t_R	retention time (elution time)
tlc	Thin - layer chromatography
mp	melting point
bp	boiling point
CDI	1,1'-carbonyldiimidazole
DMAP	dimethylaminopyridine
pmsf	phenylmethylsulphonyl fluoride

PLE	Pig liver esterase
OPE	Orange peel esterase
PPCE	Pig pancreatic cholesterol esterase
EEAcE	Electric Eel acetylcholine esterase
PPL	Pig pancreatic lipase
ANL	*Aspergillus niger* lipase
AEL	*Alcaligenes sp.* lipase
CCL	*Candida cylindracea* lipase
CLL	*Candida lypolitica* lipase
GCL	*Geotrichum candidum* lipase
MJL	*Mucor javanicus* lipase
PCL	*Penicillium cyclopium* lipase
PRL	*Penicillium roqueforti* lipase
PFL	*Pseudomonas fluorescens* lipase
RAL	*Rhizopus arrhizus* lipase
RDL	*Rhizopus delemar* lipase
RJL	*Rhizopus javanicus* lipase
RNL	*Rhizopus nivous* lipase
PLA_2	Phospholipase A_2
α-chy	α-chymotrypsin

Acknowledgements

I would like to thank Professor D.H.G. Crout for his encouragement and advice throughout the course of this work.

Thanks to everyone in the Department during my stay, especially Veronique ('allo, 'allo!), Rupert, (Action) Dan, Rock-Steady Eddy, and housemates Dave, Alan and Di.

My parents, for not asking too many awkward questions!

The gifts of enzymes by Amano Pharmaceutical Co. and Biocatalysts Ltd. are gratefully acknowledged.

Dr. T. Wallace (Salford University) is to be thanked for his advice concerning, and for generously supplying various cyclobutane esters.

Dr. A. Drake (Birbeck College), for recording the c.d. spectra.

Dr. M. Nicholds (ICI Biological Products) is to be thanked for the supply of the propanol derivative.

The financial assistance of the SERC and especially of ICI Biological Products Division is gratefully acknowledged.

to Suzie...

for putting up with me!

Declaration

The work described in this thesis is the original work of the author, except where acknowledgement is made to work and ideas previously described. It was carried out in the Department of Chemistry, University of Warwick, between October 1985 and February 1989 and has not been submitted previously for a degree at any institution.

Summary

This thesis describes investigations in the field of biotransformations, concentrating on the mode of action of esterases and lipases, and the application of these systems to the production of enantiomerically pure compounds.

The behaviour of pig liver esterase (PLE) in the presence of some commonly used alcohol and ketone cosolvents was examined. Methanol, ethanol and 2-butanone were found to be potent inhibitors of PLE. At low concentrations, acetone accelerated the rate of PLE catalysed hydrolysis, but is also inhibitory at higher concentrations. The effects of alcohols as product inhibitors were examined. This, and other data obtained suggests that the complex kinetics of this enzyme could be explained using the 'Ligand Induced Slow Transition Model' (Almalie *et al.*, J. Biol. Chem., 1972, 247, 7088). Examination of PLE by gel filtration hplc suggested that the active form of this enzyme may not be trimeric as previously thought. Some analogous studies were carried out with pig pancreatic lipase.

The use of the isozyme - specific inhibitors, eserine and phenylmethylsulphonyl fluoride did not improve the enantiospecificity of the hydrolysis of *trans*-1,2-diacetoxycyclopentane catalysed by PLE.

Dimethyl *meso*-3-cyclobutene-1,2-dicarboxylate was hydrolysed to the corresponding mono - ester by pig liver esterase with high optical purity (86% e.e.) and chemical yield (90%). The mono ester epimerised during derivatisation mediated by 1,1'-carbonyldiimidazole, allowing the preparation of optically pure *trans* derivatives. Rapid derivatisation allowed the preparation of *cis* analogues. This was demonstrated by the preparation of both the optically pure *cis* and *trans* benzylamide derivatives.

meso-3-Cyclobutene-1,2-dimethanol diacetate was hydrolysed to the corresponding mono - acetate by *Pseudomonas fluorescens* lipase with high optical purity (>97% e.e. at 0.8 mol-equivalent) and chemical yield. The corresponding (4-phenyl)benzoyl, (4-toluene)sulphonyl and (9-phenyl)xanthan-9-yl) derivatives were prepared.

The racemic allene diethyl penta-2,3-dienedioate was hydrolysed by pig liver esterase in buffer containing 25% (v/v) acetone. The product, (S)-(-)-ethyl penta-2,3-dienedioate, was produced with 30 - 32% e.e. at 0.3 mol-equivalents.

As a demonstration of the development of a hydrolytic resolution procedure, such a method was devised for the preparation of an optically pure halogenated propan-1-ol derivative. Pig pancreatic lipase was chosen as the model system. By manipulating the reaction conditions and the acid moiety, the enantiospecificity of the hydrolysis was improved by a minimum of 0.4 times, and possibly by greater than ten-fold.

Chapter 1 - Introduction

1.1.1 Biotransformations

The work presented in this thesis is concerned with the application of enzymatic catalysis to synthetic organic chemistry. This is an interdisciplinary area of research, combining elements from the traditionally disparate fields of biochemistry and organic synthesis.

The conversion of one organic substrate into another by a biological system has been designated a "biotransformation". No single definition of this term has yet been universally accepted. However, any discussion of this area of research requires such a definition and the following [1] has proved useful in practice:

Biotransformations are selective enzymatic conversions of natural or chemically synthesised substrates into defined products on a preparative scale, using whole cells or isolated enzyme systems.

The literature in this area has been well reviewed in recent years [1-6], attention will therefore be focused on certain generic problems in preparative enzymatic catalysis, and at attempts at their solution.

Many workers entering this field have a background in classical organic synthesis, and have little or no experience in

handling biological systems. They may even view biological agents as "black boxes". However, the complexity of biological systems should not dissuade a newcomer to the area, as there is already a sufficient pool of knowledge to make biotransformations a valuable weapon in the armoury of any synthetic organic chemist.

1.1.2 Applications of Biotransformations

There are four main goals in organic chemistry to which biotransformations have so far been shown to be relevant.

1. The generation of chiral synthons is possibly the single largest target of current synthetic organic chemical research. Much of the pressure directed towards this area has come from the pharmaceutical industry. This is partly based on regulatory constraints, which now favour the production of enantiomerically pure pharmaceuticals. The high degree of stereospecificity exhibited by enzymes with respect to both substrates and the reactions catalysed is the powerful tool for research in this area. The production of chiral synthons can be achieved enzymatically by one of three possible routes;

   - *Kinetic resolution*
   - *the "meso trick"*
   - *A chiral transformation on an achiral substrate*

   The latter two methods have an advantage over kinetic

resolutions of a theoretically possible 100% yield, while a kinetic resolution can only achieve a maximum of 50% of the desired enantiomer. Currently, the other main catalytic route to chiral synthons is *via* optically active transition metal catalysts.

2. Enzymes often exhibit high degrees of regioselectivity. This can be used alone, or to complement their stereospecificity. An example of this is a single, stereoselective reduction of a substrate containing more than one carbonyl group, as illustrated in Figure 1.1a [10].

3. Enzyme catalysed reactions can be easier, quicker, cheaper and/or give better results (cleaner products and/or higher yields) than conventional synthetic methods. Peptide and oligosaccharide synthesis, and remote site functionalisation are three examples. This is illustrated by the insertion of a hydroxyl group into a steroid skeleton, as shown in Figure 1.1b [11].

4. The mild conditions usually required for enzyme activity are particularly appropriate for reactions involving chemically labile substrates or products. This approach has been used successfully with sensitive esters, and an example of this is illustrated in Figure 1.1c [12].

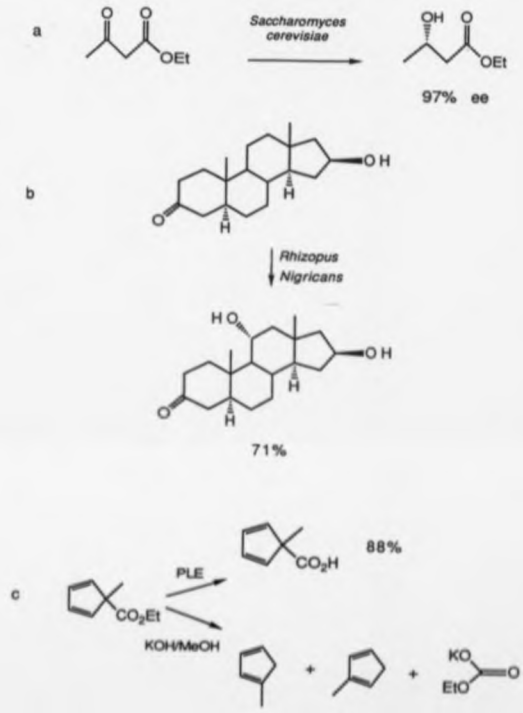


Figure 1.1 - Some applications of Biotransformations (see section 1.1.2)

1.1.3 Enzyme Characteristics Relevant to Biotransformations

As with all specialised areas, there are certain features of the system (in this case, of enzymes) that must be understood before the system can be successfully exploited. These include;

1, All reactions are catalytic. The catalyst is an enzyme or enzymes consisting of one or more proteins. Certain classes of enzymes require additional non - protein cofactors.

2, All reactions are, in theory, reversible, and will reach equilibrium if sufficient time is allowed.

3, Reactions are catalysed only over a relatively narrow temperature range. This is typically 20 - 40°C, but can be -10 - 90°C.

4, Reactions are catalysed only over a relatively narrow pH range. As with temperature, the optimum pH varies from system to system. Usually the pH is kept reasonably stationary during a biotransformation, as the catalytic behaviour of the system may vary with pH.

5, The properties of the system may vary with the form in which it is used;

- *free or immobilised*
- *whole cell, crude extract or purified enzyme*

6. Enzyme activity is subject to regulation. That is, the rate and even the course of a reaction may be influenced by the concentration of substrates, products or other components of the reaction mixture. This is usually observed as inhibition (more often than not, product inhibition), although activation is sometimes observed.

7. The catalytic cycle may well alter the form of an essential cofactor. Therefore, cofactor recycling and / or the addition of NADH, NADPH or ATP may be required. This applies mainly to isolated redox enzymes. The use of stoichiometric amounts of such cofactors is usually prohibited by cost. The simplest solution to this problem is the use of whole cells.

8. Biological systems have a limited operational lifetime. This could be due to, for example, cell lifetimes, toxicity in whole cell systems, or enzyme degradation. The latter is especially true for crude preparations, which may well contain proteases.

Other factors, although not characteristic of biological systems, can also have an important influence on their use. Such factors include their availability and cost.

1.1.4 Enzyme Classification

There is an enzyme catalysed equivalent to virtually all classes of reactions utilised in synthetic organic chemistry. It was thought that the Diels - Alder reaction and the Cope rearrangement were major exceptions, but there is now evidence that these too are catalysed.

The International Union of Biochemistry classification scheme assigns all known enzymes to one of six possible groups;

1, Oxidoreductases

Enzymes in this class catalyse oxidation - reduction reactions involving oxygenation, or overall removal or addition of hydrogen atom equivalents.

2, Transferases

Enzymes in this class catalyse the transfer of groups from one molecule to another. Such groups include acyl, sugar, phosphoryl, aldehyde and ketone moieties.

3, Hydrolases

Enzymes in this class catalyse the hydrolysis of carbon - heteroatom functional groups. Such groups include esters, anhydrides, glycosides, amides and peptides.

4, Lyases

Enzymes in this class catalyse additions to double bonds

and eliminations to form double bonds. The addition / elimination moiety is usually of the form HX. The types of double bonds involved includes C=C, C=N and C=O.

5. Isomerases

Enzymes in this class catalyse a variety of isomerisations. These include C=C bond migrations, cis - trans isomerisations and racemisations.

6. Ligases

Enzymes in this class catalyse the formation of a variety of bonds. These include C-C, C-O, C-N, C-S and phosphate ester bonds. Enzymes in this class are often referred to as synthetases.

Currently, enzymes of classes 1, 3 and 4 have proved to be the most useful for biotransformations. Enzymes of classes 2 and 6 are generally too substrate specific for general use. The isomerisations of class 5 are often easily accomplished chemically.

Only enzymes of class 3 have been used in the work presented in this thesis.

1.1.3 A Biotransformations Census

Any survey of the biotransformations literature will throw up examples of the use of exotic biological systems and novel procedures. However, it would also reveal that a small number of

biological systems account for the great majority of publications.

To illustrate this point, one such survey was carried out using the papers and patents included in the *Warwick Biotransformation Abstracts* [13] for the period 1987 - 88. Publications are selected for these abstracts using the criteria that they should be of interest to synthetic organic chemists and that they should fall broadly in line with the definition of biotransformations given in section 1.1.1.

The frequency of reaction types found was:

40% *esterolytic*

29% *hydrogenase mediated*

24% *oxygenase mediated, peptide and oligosaccharide synthesis*

3% *carbon - carbon bond formation*

4% *all other types*

The esterolytic types of reaction (comprising hydrolysis, synthesis and transesterification) were subdivided as follows:

29.3% *lipases*

8.4% *esterases*

6.4% *proteases*

Pig liver esterase (PLE) dominates the esterase group at 82%. This is due in large part to the fact that very few true esterases are commercially available.

Despite the fact that a large number of lipases are available, and that this number is increasing steadily, the lipase group is dominated by just two systems. These are Pig Pancreatic lipase (PPL) and *Candida cylindracea* lipase (CCL) with 40% and 29% respectively of the lipase applications.

The most prevalent system in the hydrogenase group is bakers' yeast (*Saccharomyces cerevisiae*), at 54% of the hydrogenase applications.

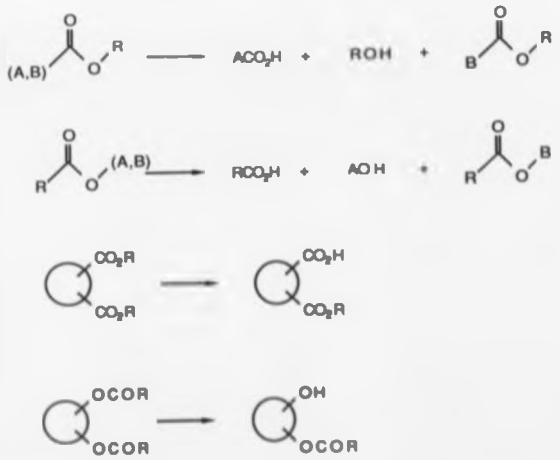
From this, it can be seen that a small number of biological systems (currently four) dominate the field of biotransformations, with over one-third of all published applications during 1987 - 88.

1.2 Some Aspects of Enzymolytic Biotransformations

1.2.1 Stereospecific Hydrolysis Reactions

In addition to esterases and lipases, proteases are also capable of hydrolysing esters. Chiral products can be produced either *via* classical kinetic resolutions of racemic esters, or *via* enantioselective hydrolyses of *meso* esters containing at least two ester groups.

The ability of some enzymes to distinguish readily between chiral centres in *meso* compounds is an extremely useful property. There is no general method analogous to this in conventional synthetic chemistry, and as this procedure has been labelled the "meso trick".



where I) R is achiral, and
II) A and B are the enantiomers of a chiral moiety

Figure 1.2 - Schematic representation of the routes to chiral products from the hydrolysis of esters

Both of these mechanisms can be further subdivided on the basis of the position of the chiral centre(s) of interest within the substrate ester. Thus, a kinetic resolution could be of a chiral carboxylic acid or of a chiral alcohol, and a meso trick could be used on, for example, a meso - dicarboxylic acid diester or on a

meso - diol diester. All four routes are illustrated schematically in Figure 1.2..

Optical purities are usually quoted in terms of the enantiomeric excess (e.e.), where:

$$e.e. = \frac{\text{major enantiomer} - \text{minor enantiomer}}{\text{major enantiomer} + \text{minor enantiomer}}$$

It is usually quoted as a percentage, where 0% is a racemate, and 100% is one enantiomer.

1.2.2 The *meso* Trick

Usually only one enantiomer of a chiral synthon is required. Therefore, kinetic resolution of racemates should be avoided whenever possible. Even when the unwanted enantiomer can be recycled, this introduces extra stages into a process. *meso* compounds possess the symmetry elements (S symmetry) that permit total asymmetric induction by enzymes, thus circumventing the problem. The use of the *meso* trick is one of the most successful applications to emerge from biotransformation research.

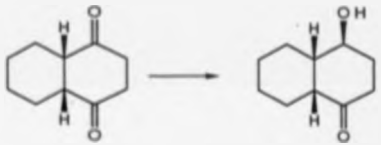
The great majority of *meso* trick applications have been on cyclic substrates, although there are many notable acyclic applications. Alcohol dehydrogenase (mainly from horse liver) has found wide application in the stereospecific oxidation of *meso* diols and, to a lesser extent, reduction of *meso* diketones. Some examples of

ref. [14]

90% yield
100% ee

ref. [14]

79% yield
100% ee



ref. [15]

54% yield
100% ee

Figure 1.2 - Some "meso trick" reactions using alcohol dehydrogenase

this are illustrated in Figure 1.3. Stereospecific hydrolyses of meso - diesters (both dicarboxylic and diol esters) by PLE, PPL and *Pseudomonas Fluorescens* lipase (PFL), amongst others, are well

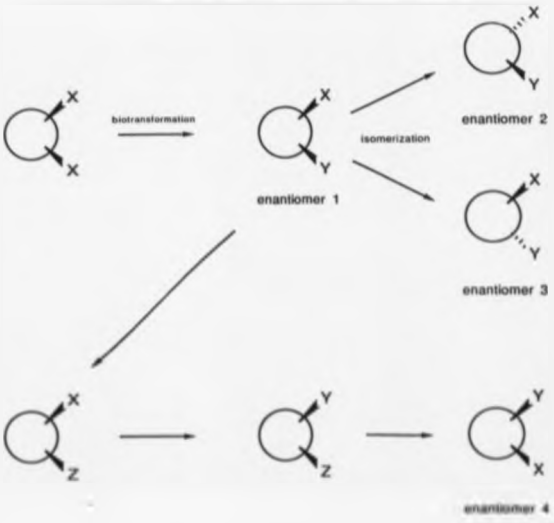


Figure 1.4 - Some routes to all four possible enantiomers of cyclic meso substrates

known. (For examples, see sections 1.3.1, 1.4.2 and 1.4.3).

Functional group manipulations on the products of meso trick reactions can, in principle, give access to either of the two enantiomers possible from a meso substrate. Thus, even if a meso trick reaction gives the unwanted enantiomer, the required

enantiomer may still be produced chemically.

In the case of cyclic compounds, this principle can be extended, to generate one or either of the two remaining enantiomers possible for two chiral centres. This is *via*, chemistry willing, a controlled *cis* - *trans* isomerisation at one of the two relevant functional groups and, if necessary, further functional group manipulations. This can act as an alternative to carrying out the biotransformation on the *trans* isomer, which is by definition, racemic. Some combinations of these manipulations are illustrated schematically in Figure 1.4.

1.3.3 A Predictive Model for Hydrolytic Resolutions

Most hydrolytic enzymes used for biotransformations possess a serine residue at their active sites, which is essential for activity. They function by binding the substrate ester (amide, etc), and hydrolysing it. An acyl - intermediate is formed and the alcohol component (P_1 in equation 1) is released. This intermediate is formed *via* the hydroxyl group of the active site serine residue, and is cleaved by attack from a nucleophile (usually water). This releases the other product (P_2). This cycle is summarised in a schematic form in equation 1.

eq.1

In order to achieve a resolution, it is necessary for one enantiomer to react faster than the other. This could be brought about either *via* stronger binding or *via* faster catalysis, or both. If it is assumed that the discrimination occurs at the binding stage, then the substrate enantiomers will act as competitive inhibitors of each other. As such a reaction proceeds, the concentration of the faster reacting enantiomer will decrease, allowing greater reaction of the slower reacting enantiomer. On the basis of these assumptions, a kinetic model has been proposed [10], which allows various parameters to be calculated. The model defines a parameter called the "enantiomeric ratio", or E, where:

$$E = \ln(A_t/A_0) \;/\; \ln(B_t/B_0) = (V_A/K_A) \;/\; (V_B/K_B)$$

eq.2

A and B represent the two enantiomers at times t and 0, and (V/K) is the specificity constant for an individual reaction. E is related to the extent of reaction (conversion ratio, c), as defined in equation 3;

$$E = \ln[1-c(1+ee_p)] \;/\; \ln[1-c(1-ee_p)]$$

eq.3

The value of E should be a constant for any value of c. The interrelationship of E, c and the e.e.'s of substrates and products allows prediction of the conversion ratio required for the

optimum product or substrate e.e. from a single experiment. The higher the value of E, the greater the yield with optimum e.e.. At lower values of E, good e.e.'s can still be achieved, but this will be at the expense of yield. The model also allows for a compromise between yield and e.e. to be determined.

Despite the assumptions implicit in this model, good agreement is usually observed between predicted and observed results. These types of models can also be developed for *meso* trick reactions, and for transesterifications in two - phase [17,18] and low - water systems [19].

1.3 Pig Liver Esterase

1.3.1 Some Hydrolytic Reactions

The range of substrates accepted by PLE is remarkable. They include a highly water - insoluble alkenic ester (1) and an organometallic diene complex (2). The latter exhibits planar chirality, and was the first such resolution to be achieved enzymatically. Some other examples of the use of PLE as a biotransformations catalyst are illustrated in Figures 1.5 - 1.7.

Resolutions of chiral alcohols *via* PLE catalysed hydrolysis of esters are much rarer than for chiral acids. Of the examples given in Figure 1.6, there is a decline in optical purity of the diol product when increasing the ring size from four (3) to five (4). The optical purity then improves when the ring size is further increased to six (5), but the diol product is one of the

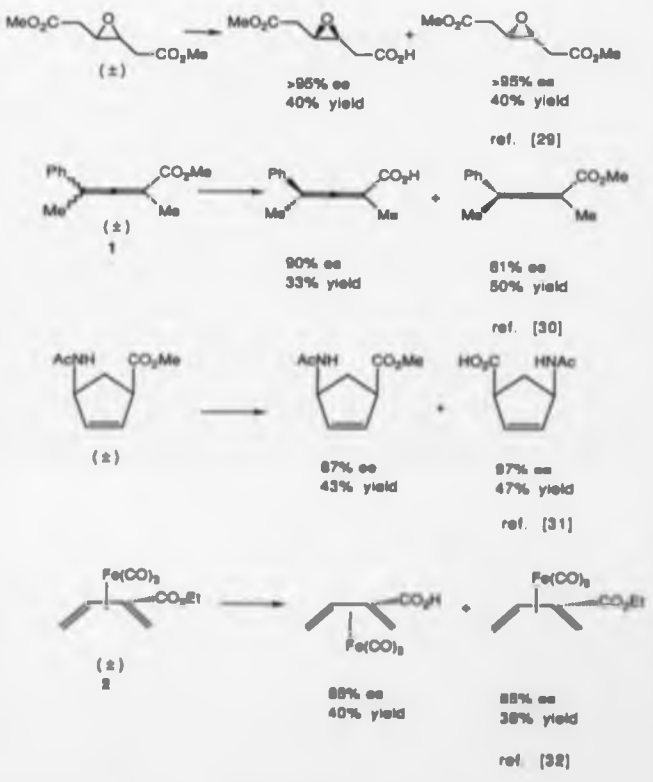


Figure 1.8 - Some resolutions of chiral acid esters achieved with PLE

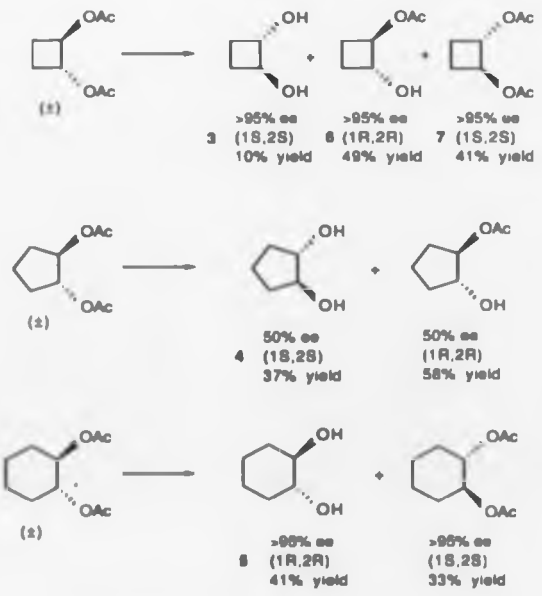


Figure 1.6 - Some resolutions of chiral alcohol esters achieved with PLE

opposite configuration. This is a widely observed phenomenon for the PLE hydrolyses of a series of related esters [20-28].

Another interesting observation is that the cyclobutane

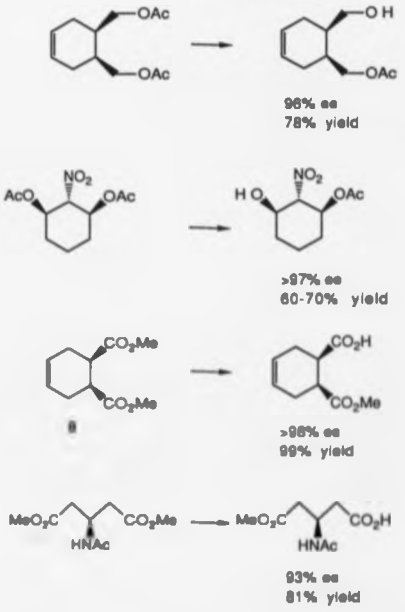


Figure 1.7 - Some "meso trick" hydrolyses achieved with PLE

diol 3 has the same configuration as the unreacted diacetate 7. This indicates that the monoacetate formed from the hydrolysis of 7 is a better substrate for further hydrolysis than is the more

rapidly formed monoacetate 6.

There are many examples of PLE hydrolyses of *meso* diesters. Perhaps the most widely known example is the now classic hydrolysis of 8. This reaction is virtually quantitative with respect to both yield and optical purity. Further examples of the hydrolysis of cyclic *meso* dicarboxylic acid esters can be found in chapter 5. As with racemates, reports involving *meso* alcohols are rarer than for *meso* carboxylic acids.

1.3.2 Pig Liver Esterase as a Biocatalyst

PLE is an attractive enzyme for effecting biotransformations. It is readily available, reasonably priced, requires no expensive auxiliaries and accepts a wide diversity of substrates whilst retaining stereospecificity.

However, this situation is not mirrored in the enzymology of the system. Preparations of PLE are mixtures of isozymes [39-42], which appear to be closely related. They have slightly different, but overlapping physical characteristics. Until the successful separation and characterisation of some of the isozymes was achieved [41,42], the results from different laboratories appeared to defy explanation. Until the early 1970's, even the molecular weight of the monomer was disputed [43,44].

It was subsequently discovered that one of the problems was related to the substrates used to monitor enzyme activity during purifications. Several different substrates were used in various laboratories. This led to apparently homogeneous preparations, which

were actually enriched in certain of the isozymes. The isozymes enriched depended on the substrate chosen to follow enzyme activity. Thus, data on "homogeneous" preparations from different groups was inconsistent and sometimes contradictory.

This "microheterogeneity" could have several origins. Each isozyme might result from its' own unique gene, which may form part of a multigene family. Such families can arise by gene duplication, followed by mutation. Alternatively, there might be a single gene. Isozymes could then arise from modification of either the messenger RNA (post - transcriptional modification), or of the peptide itself (post - translational modification).

The first report of the cloning and sequencing of a cDNA that encodes a mammalian liver carboxylesterase has recently been published [45]. This was the rat liver enzyme, but all mammalian esterases appear to be closely related [46,47]. The authors concluded from Southern Blot analysis that this enzyme is part of a multigene family.

The "microheterogeneity" is further complicated by variations in the relative proportions of the isozymes found in individual animals [41].

1.4 Lipases

1.4.1 Differentiation from Esterases

The difference between a lipase and an esterase is

usually defined by operational conditions. The physiological substrates of lipases are glycerides [48,49], and lipase activity is typically assayed on the basis of the ability to hydrolyse long - chain (C_{10-18}) fatty acid esters of glycerides. Esterase activity is expressed as the ability to hydrolyse short - chain (C_{1-4}) esters, either of glycerides or of simple esters. Clearly, this type of definition is ambiguous.

A less ambiguous definition is based on the phase behaviour of the optimal substrates. That is, the short - chain esters used for esterase determinations will be monomerically dispersed in aqueous media (i.e. truly in solution). Long - chain glyceride esters exhibit low water solubility, and have a tendency to aggregate into micelles or to form emulsions. This produces a non - homogeneous reaction system. True lipases concentrate at, and are stabilised by such interfaces. They are also catalytically highly active at interfaces, when compared to acting on dissolved substrates [50,51]. True esterases exhibit maximum activity against dissolved substrates.

As many organic compounds have low water solubilities, the interfacial behaviour of lipases has proved extremely useful. Many lipase - catalysed hydrolyses have been performed in two - phase systems. These consist of an aqueous component containing the enzyme, and an immiscible low polarity [52] solvent (typically hexane or isooctane) containing the substrate. On mixing, a large interfacial area is produced, leading to enhanced reaction rates. This experimental design has an additional advantage, in that the product acid may partition into the aqueous phase by forming a water

soluble salt with a buffer component. This may displace the equilibrium and also simplify the subsequent work - up.

1.4.2 Some Biotransformations with *Pseudomonas fluorescens* lipase

The lipase from *Pseudomonas fluorescens* (lipase P, Amano, PFL) has recently been used to catalyse the hydrolysis of cyclic mono- and diol esters. Some examples are shown in Figure 1.8. This lipase preferentially hydrolyses acetates of the *R* configuration. An exception to this observation is *meso*-1,2-diacetoxycyclopentane (9) , which gives the *S*-alcohol with very high optical purity [53].

The highly specific hydrolysis of *trans*-1,2-diacetoxy-cyclopentane (10) is an alternative to the reaction catalysed by PLE, which displays only moderate enantiospecificity in this case (see Figure 1.6).

1.4.3 Some Biotransformations with Pig Pancreatic Lipase

Some examples of the use of PPL as a catalyst for biotransformations are illustrated in Figures 1.9 (resolutions) and 1.10 (*meso* hydrolyses).

Typically, lipases are more specific towards hydrolyses of chiral alcohol esters, than towards esters of chiral acids.

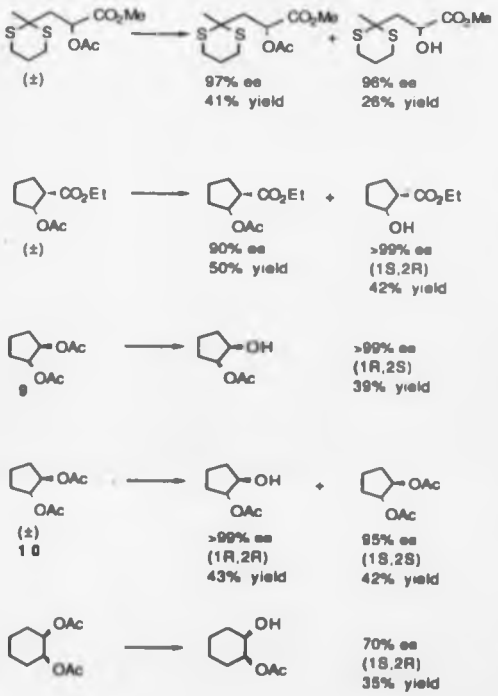


Figure 1.8 - Some hydrolyses with PFL

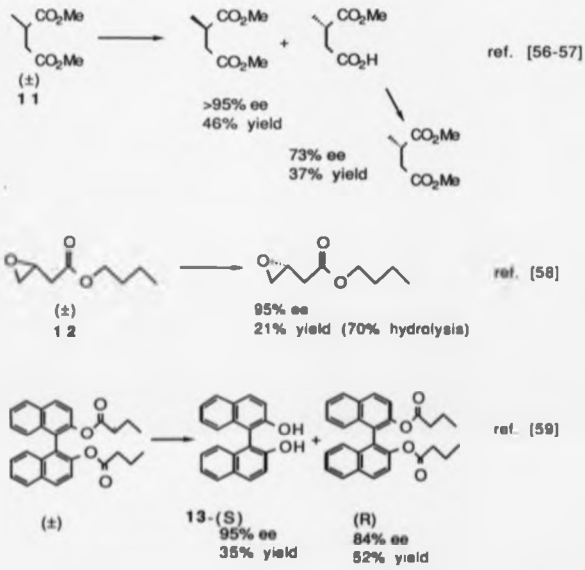


Figure 1 9 - Some resolutions with PPL

Despite this, PPL has been used successfully in the resolution of several chiral acids. These include α-substituted succinate esters (11) and an epoxy ester (12).

The binaphthol 13 is useful in the preparation of chiral reducing agents, crown ethers and organotitanium reagents.

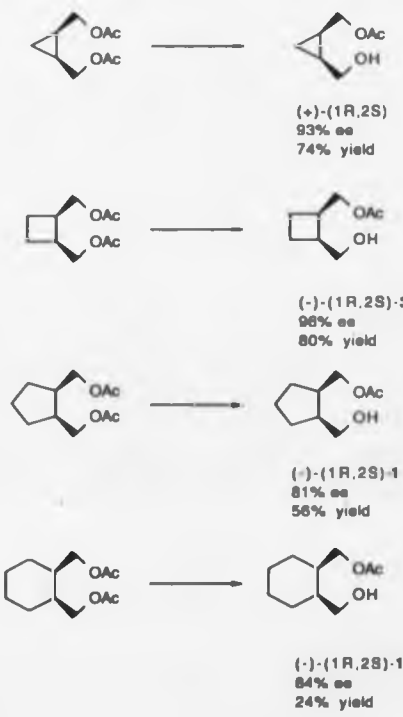


Figure 1.10 - Some "meso trick" hydrolyses with PPL [60-61]

The optically pure (S)-diol was produced *via* PPL hydrolysis of the valerate diester. The extremely low water solubility of the substrate was overcome by the use of a multiphase reaction system with polyvinyl alcohols added as emulsion stabilisers.

The hydrolysis of the cyclic *meso* diacetates illustrated in Figure 1.10 occurs rapidly, even in the absence of a non - aqueous phase. All four products can be prepared with high optical purities, although in the case of 15, and to a lesser extent, 14, this is at the expense of yield.

1.4.4 Steapsin as a Biocatalyst

Commercial PPL preparations, such as "Steapsin" and "Pancreatin" are very impure, containing many other enzyme activities in addition to that of the lipase (see section 6.2.1). One such preparation, type II lipase (Sigma), contains both amylase and protease activity, and is only 17 - 35% protein. Several different grades of purity of PPL are available. Clearly, one may wish to "improve" the biotransformation by using a preparation of higher activity and purity. However, when this was attempted, the rate and enantioselectivity of the reactions decreased ([62] and section 5.2.3). This demonstrates that some enzyme other than the predominant lipase is the chiral catalyst of interest in these cases.

In the case of a partially stereospecific reaction with a crude PPL preparation, the incomplete specificity could be due to an inherent feature of a single hydrolysing enzyme, or a result of

competing enzymes with different enantiospecificities towards the substrate. These preparations contain significant serine protease levels, which are capable of ester hydrolysis. To date, three techniques have been applied to overcome this problem. It is possible to selectively inhibit the serine proteases by the use of a specific inhibitor such as p-toluenesulphonyl fluoride (pmsf) [63]. Alternatively, the preparation could be fractionated to purify the desired stereospecific activity [64]. The final approach relies on the unique interfacial characteristics of lipases; If a two - phase reaction system is employed, then only the interfacial enzymes will "see" the substrate, thus preventing non - specific protease catalysed hydrolysis.

Chapter 2 - Pig Liver Esterase
- Some Physical Studies

2.1 Introduction

Knowledge of the physical properties of a catalyst may not be required for preliminary experiments, but the insight that such knowledge brings is usually essential inorder to progress beyond initial results. This is as true for pig liver esterase (PLE) as for any other enzyme. For while it may not be essential to know the relevant rate constants in order to carry out a simple hydrolysis, knowledge of PLE's cosolvent and immobilisation characteristics will greatly assist any optimisation work.

Much of the confusing biophysical data on PLE has now been clarified by the partial separation and characterisation of isozymes [41,42]. This is little use, however, to practitioners of biotransformations, since they will not be using homogeneous isozyme preparations. Instead, they will be using the same "mixed isozyme" systems that led to the original confusion. This is the system that they will require physical data for. Therefore, it was thought relevant to repeat, extend and re - assess some of the physical studies on PLE which bore some relevance to biotransformations.

It has long been known that PLE displays complex kinetics [63,66] and a number of models have been advanced at different times. Results have been obtained in this work, consistent with previous data, suggesting that PLE may conform to a standard kinetic

model for monomeric enzymes that exhibit non - Michaelis Menten behaviour.

One of the features which distinguishes the various isozymes making up PLE is their susceptibility to inhibition. Work on such differential inhibition has previously focused only on the purified isozymes [67]. In this work, a study was made on the mixed isozyme system. This was to see if one isozymal activity could be inhibited while leaving other activities intact. This could have consequences if, for example, an unwanted side reaction was found to be catalysed by a specific isozyme population. Such a reaction could then be prevented.

Gel filtration hplc is a rapid and convenient method for determining the molecular weights of proteins. Although less accurate than electrophoretic methods, hplc allows the direct observation of intact multimeric proteins and, under appropriate conditions, may reveal changes in the distribution patterns of oligomers and their subunits.

Biotransformations utilising PLE are typically, but by no means exclusively, carried out in water. Unlike lipases, PLE is sensitive to interfaces (being adversely affected). Because of this, reactions are usually performed in homogeneous systems. Many of the substrates that make ideal candidates for PLE catalysed biotransformations have low water solubilities. To achieve adequate reaction rates in this situation the substrate must somehow be brought into intimate contact with the enzyme. This is often achieved by the use of water - soluble cosolvents. The addition of cosolvents to a reaction can have a variety of effects. These can

range from altering the reaction rate or displacing the equilibrium, to altering the (typically stereochemical) outcome of a reaction.

Since enzymes are simply catalysts, they can, in principle, be used repeatedly. Where two - phase reaction systems are used, and the desired reaction components partition into the non - aqueous phase, the phases can just be separated. This allows the enzyme - containing aqueous phase to be recycled. However, two - phase systems are rare with PLE. For dissolved enzymes in homogeneous reaction systems, reuse of the catalyst is not possible. This is because recovery of the reaction components is achieved by vigorous solvent extractions. Such procedures denature and precipitate proteins.

The recycling of enzymes can be achieved by binding the enzyme to an insoluble polymer, thus immobilising it. The catalyst can then be recovered by filtration. Not only does immobilization improve the economics of a biotransformation, but it also makes it possible to terminate reactions at exactly the point required. However immobilised enzymes rarely exhibit activities as high as their soluble counterparts. Therefore a balance has to be struck between the activity lost on immobilisation and the number of times the catalyst can be reused.

2.2 Results and Discussion

2.2.1 Kinetics and Cooperativity

The assay for esterase activity used throughout this work was based on that devised by Dudman and Zerner [68], with minor modifications. This assay has several advantages over some others in the literature; No corrections are required for spontaneous hydrolysis, CO_2 absorption or incomplete butyric acid ionization, and importantly, no cosolvents are required for substrate dissolution. The use of cosolvents can mask kinetic features such as substrate activation.

The rate of hydrolysis of ethyl butyrate increases linearly with substrate concentration between 0.5 and 10 mM (see Figure 2.1). When, however, the data is transformed to either the Lineweaver - Burk (1/[s] v. 1/[v]) or Eadie - Scatchard (v v. v/[s]) formats, the data is clearly biphasic (see Figure 2.2). Both sections of data are linear, and K_m and V_{max} values can be deduced. These have been termed K_L and V_L for the low concentration range, and K_H and V_H for the high concentration range. The values are listed in Table 2.1.

The biphasic nature of PLE catalysed hydrolyses has been observed previously [65,66,69]. Dudman and Zerner [68], and Greenzaid and Jencks [70] make no comment on this situation, giving only a single value for K_m (0.5 and 0.6 mM respectively). These are close to the value obtained for K_L.

The non - Michaelis Menten behaviour of PLE has

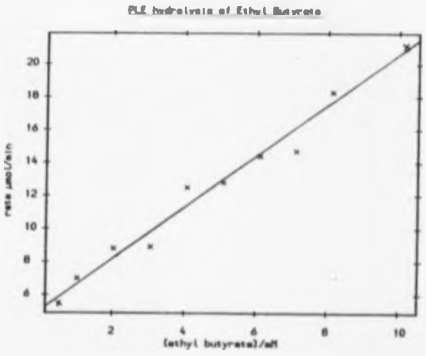


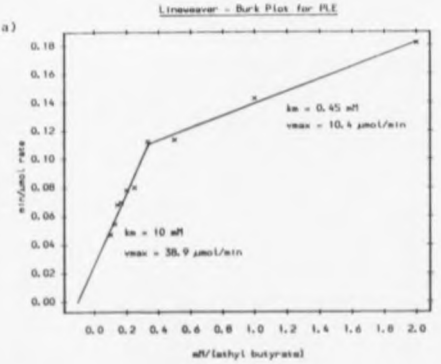
Figure 3.1

	Lineweaver - Burk	Eadie - Scatchard
K_H	10 mM	13.9 mM
V_H	39.9 µmol/min	48.9 µmol/min
K_L	0.45 mM	0.48 mM
V_L	10.4 µmol/min	10.6 µmol/min

Table 3.1

previously been explained on the basis of a two site mechanism. Kistiakowsky and Adler [71,72] proposed that two sites were present; One high affinity / low velocity site and one low affinity / high velocity site. This model was, however, ruled out on the basis of

a)


Lineweaver - Burk Plot for PLE
km = 0.45 mM
vmax = 10.4 µmol/min
km = 10 mM
vmax = 38.9 µmol/min
mM/(ethyl butyrate)

b)

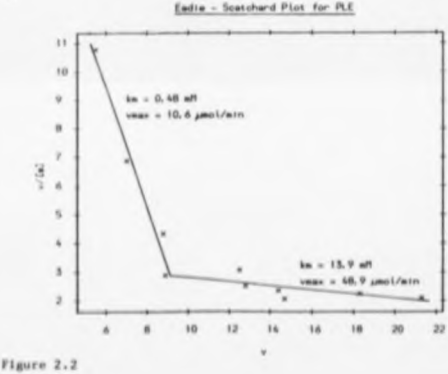

Eadie - Scatchard Plot for PLE
km = 0.48 mM
vmax = 10.6 µmol/min
km = 13.9 mM
vmax = 48.9 µmol/min
v

Figure 2.2

inhibition studies. Instead, a single active site, with a separate "control" site or sites was suggested [66,69,73]. It has been clearly shown that subunit interactions are not required to obtain these kinetic features [73]. This means that classical Monod - Changeux - Jacob type allosteric interactions are not responsible for the observed results. Studies on the kinetics of inactivation of PLE using bis (p-nitrophenyl)phosphate monoanion [73] have shown that there is some form of heterogeneity in addition to the complex isozymal patterns observed with isoelectric focusing. As a consequence of this, it was proposed that the isozymes can exist as at least two different conformers, that the conformers can interconvert only slowly, that the conformers have different kinetic parameters, and that the interconversion of the forms is mediated by modifiers such as phenol and ethyl thiolacetate. Under the experimental conditions employed, it was further suggested that the modifiers could not be bound at the active site. Rather, modifiers were only bound at the so - called "control" site.

All of the features of this model, along with some other kinetic observations (such as pre - steady state burst and lag phases) fit neatly into the requirements of the *Ligand Induced Slow Transition Model* [74,75]. This model predicts that either positive, negative or no cooperativity may be observed under appropriate steady - state conditions for monomeric enzymes existing in slowly interconverting forms, and either burst - type or lag - type transients may be found.

The data presented in Figure 2.2 was transformed to the format of the Hill plot (see Figure 2.3) to see if cooperativity was

evident in this work. The gradient of the best straight line obtained (the Hill coefficient, n) is taken as a measure of the minimum number of interacting sites. Where n = 1, no cooperativity is present. The Hill plot shows that cooperativity was not observed under the experimental conditions employed. In addition, although burst and lag - type pre - steady state features were observed with some substrates used in this work, these were never found with ethyl butyrate. It should be emphasized that the presence or absence of cooperativity would not, on its' own, prove either way the validity of the kinetic model proposed.

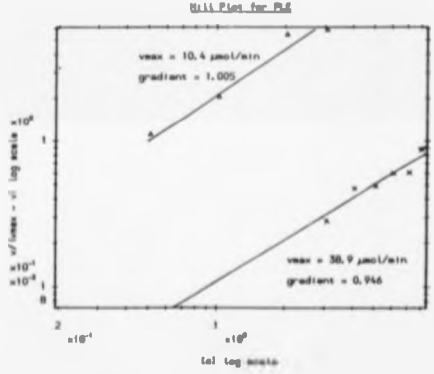


Figure 2.3

2.2.2 Cosolvents and Alcohols as Product Inhibitors

The effects of four water - miscible organic solvents on the rate of PLE catalysed hydrolysis of ethyl butyrate were examined. One solvent, acetone, produced a marked increase in the reaction rate at low concentrations (2-6% v/v). The other solvents all reduced the observed reaction rates (see Figures 2.4 and 2.5).

The rate accelerating effect of acetone is well known [67,70,73]. The maximum rate enhancement found by Barker and Jencks [73] was 175 % at 0.1 M, using p-nitrophenyl acetate as substrate. This value is in good agreement with that found in this work (164 % at 3.4 % v/v).

Biotransformations are typically carried out at elevated temperatures. This, coupled with the low boiling point and high volatility of acetone means that its concentration is continuously decreasing. Over the short assay period used (7 minutes) this was not evident. However, for longer reactions, and especially where the acetone is being used to dissolve a water - insoluble substrate, this is a problem. The addition of acetone aliquots during the reaction does not work for hydrolyses involving titration. This is because acetone, in common with many solvents, alters the pH_{app}, thus giving false titration values.

Because of the similarity between 2-butanone (methyl ethyl ketone) and acetone (dimethyl ketone), it was thought that 2-butanone might also enhance the reaction rate, while not evaporating at the reaction temperatures employed. Unfortunately 2-butanone was purely inhibitory.

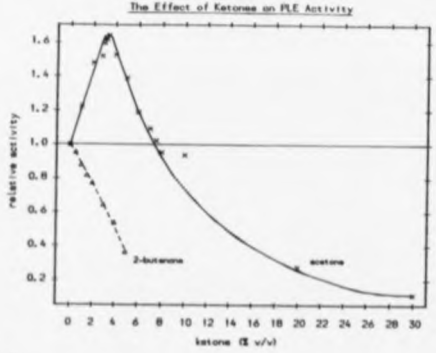


Figure 2.4

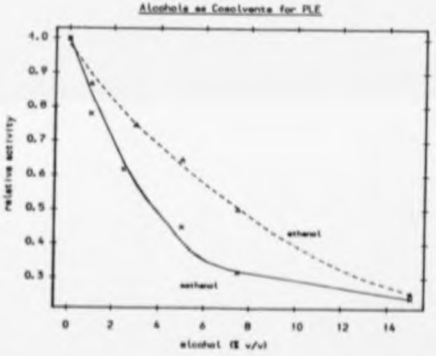


Figure 2.5

It has been shown that the low and high isoelectric point isozymes (so - called isozymes I and V) of Haymann and Junge [41] behave very differently towards acetone. Isozyme I shows rate enhancement of 150 - 160 %, while isozyme V shows only inhibition [67].

Ethanol and methanol were found to act as inhibitors in the hydrolysis of ethyl butyrate. It has long been known that methanol and other weakly acidic alcohols are effective nucleophiles towards the acyl - enzyme intermediates in PLE catalysed reactions [70]. Methanol can be a better nucleophile than water, bringing about a five - fold increase in the rate of disappearance of phenyl acetate, without greatly affecting the hydrolysis rate. However, in this work the hydrolysis rate was decreased. This was presumably because of the competition between the alcohol and water at the active site. The total rate of ethyl butyrate disappearance was not determined.

Methanol was found to be a stronger inhibitor of ethyl butyrate hydrolysis than was ethanol. This could be due to a variety of factors, including competition between the two alcohols as nucleophiles. In order to simplify the situation, PLE hydrolysis in the presence of alcoholic cosolvents was examined with the corresponding butyrate esters as substrates. Not only does this type of study suggest suitable concentrations for cosolvents, but it also hints at the potential for scale - up. That is, increasing the concentration of reactants may be limited to a level where the build - up of alcohol during a hydrolysis does not detrimentally affect the reaction rate. Figure 2.6 shows that methanol is a significantly

stronger product inhibitor than is ethanol. Esters of 2-chloroethanol have been suggested as suitable substrates for PLE catalysed hydrolyses because of enhanced hydrolysis rates compared to methyl and ethyl esters. While the hydrolysis rate was greater (data not shown), 2-chloroethanol was also a much more potent inhibitor than either methanol or ethanol. For this reason, hydrolysis of 2-chloroethyl esters should be limited to low product concentrations.

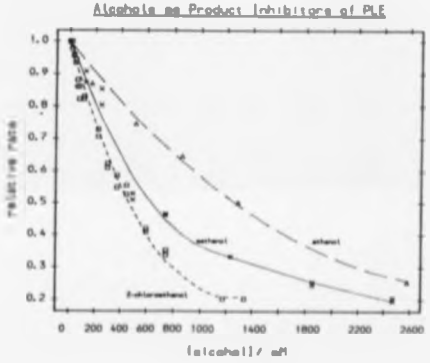


Figure 2.6

2.2.3 Inhibition of Specific Isozymes

Previous data on the use of isozyme specific inhibitors with PLE focused on the incubation times required for inhibition of the purified isozymes [67]. This work looked at the effects of inhibitor concentrations on crude PLE (the commercially available mixture of isozymes). It can be seen that phenylmethylsulphonyl fluoride (pmsf) has little or no inhibitory effect below approximately 50 μM (see Figure 2.7). There is then a rapid decrease in activity up to 500 μM. The residual activity (35 %) appears resistant to inhibition by pmsf at concentrations in excess of 1 mM.

Using the nomenclature of Heymann and Junge [61], isozyme V (the high pI isozyme) is sensitive to pmsf inhibition, retaining only 6 % activity after incubation for 30 minutes with 10 μM pmsf [67]. Isozyme I is relatively resistant to pmsf, exhibiting 73 % residual activity under the same conditions.

Eserine (physostigmine) causes a small loss of activity (10 %) at very low concentrations (<5 μM). There is no further inhibition until the eserine concentration exceeds 100 μM. After this, the residual activity decreased, and did not level off below the highest concentration assayed (1 mM).

Junge and Heymann [67] report that isozyme V is completely resistant to eserine inhibition at 10 μM (3 minute incubation), whereas isozyme I has only 3 % residual activity. This suggests that the plateau in inhibition between 5 - 100 μM is due to complete inhibition of isozyme I and no inhibition of isozyme V. At least three different types of monomer are known to constitute PLE

(α, β and γ). Isozyme V is thought to be a homogeneous oligomer of α monomers. Isozyme I is not homogeneous for γ, but contains small amounts of the β monomer. The other isozymes (II - IV) are thought to be hybrids graduating in subunit composition from I to V. The properties of β have not yet been deduced, but it is thought to be grossly similar to γ. Therefore, two possibilities exist for the reduction in PLE activity at >100 μM eserine. One is that, at high concentrations, isozyme I (and by implication, γ) is susceptible to eserine inhibition. The other is that between 5 - 100 μM eserine, both β and γ are resistant, and at higher concentrations γ remains resistant while β is inhibited. The converse of the latter argument would be pure conjecture, as there is no evidence relating to the inhibition characteristics of β.

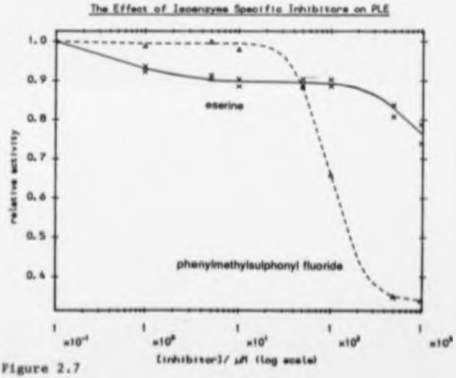


Figure 2.7

2.2.4 Gel Filtration Data

There are many suggested plots for calibrating TSK G - type columns [76,77]. One of the simplest, log(mw) v. t_e, proved adequate in this work, with a linear correlation in excess of 0.99 (7 data points, see Figure 2.8). From this, the major peak for PLE at pH 7.5 has a molecular weight of 122.6 - 124.4 kd (see Figure 2.9). There is a second peak of 54.6 - 55.7 kd. The molecular weight of both the monomer and the aggregated state was, for a long time, disputed [43,44,65,68]. However, the consensus is now that the monomers have a molecular weight of 55 - 60 kd [41,43,44]. The value of the second peak is within this range. The major peak is approximately double this figure, and so is likely to be a dimer. This conclusion is at variance with the current dogma, which states that PLE is predominantly trimeric. The current dogma is based largely on ultracentrifugation measurements. Using the calibration plot obtained, a trimer (165 - 180 kd) would be expected to elute at t_e = 30 ± 0.5 minutes. At pH 7.5 there is a small, only partially resolved peak at approximately 29 minutes. This corresponds to a molecular weight of 195 kd, which if this is a higher form, would be 3.25 - 3.5 monomers.

Despite the opinion expressed by Heymann *et al.* [43] that PLE is trimeric, their paper contains evidence that this is not the complete story. They obtained a dimer of 121.6 kd by disc electrophoresis, with only a minor trimeric band. Junge *et al.* [44] obtained a hexamer (317 kd) by electrophoresis. They stated that "evidence for a dimeric or tetrameric form of PLE was never

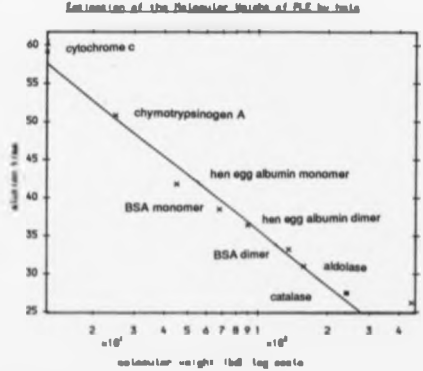


Figure 2.8

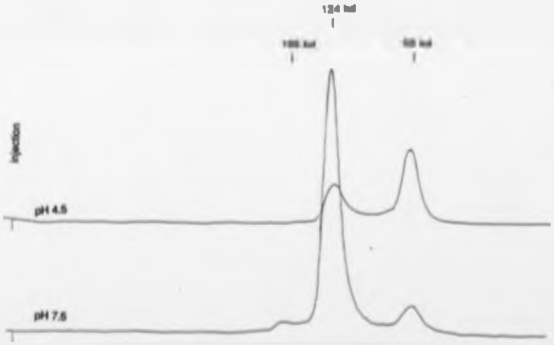


Figure 2.9 - Gel filtration hplc chromatograms for PLE

obtained". This is despite the fact that they obtained both dimers and trimers in crosslinking experiments using dimethyl suberimidate. Naumann *et al.*, commenting on their conclusions, state that "It is difficult, however, to reconcile the assumption of three subunits with the reported number of two active sites" [78,79].

Before further conclusions were drawn, it was thought necessary to look at some features of the hplc chromatogram obtained. It is known that both monomeric and higher forms of PLE are catalytically active [73] and that cooperativity is absent (see section 2.2.1). Additionally, PLE can be readily and reversibly dissociated into monomers [65,68,80]. It was thought important to demonstrate these characteristics for the chromatogram of PLE.

To check that both peaks obtained were esterase, fractions were collected. Each was analysed for ethyl butyrate and p-nitrophenyl acetate [81] activity, and the protein content was measured using the Coomassie Brilliant Blue G-250 assay [82]. The results are summarised in Figure 2.10. The specific activity calulated was for ethyl butyrate hydrolysis. It can be seen that there are two levels of specific activity, one of 173 corresponding to the dimer, and one of 110 corresponding to the monomer. This difference in specific activity for the two forms may, at first, seem surprising - especially given that it is not due to cooperativity. However, this could be explained in several ways using the *Ligand Induced Slow Transition Model* (see section 2.2.1). Although PLE displays different activities towards ethyl butyrate and p-nitrophenyl acetate, it can be seen that the ratio of activities remains constant for all fractions. This

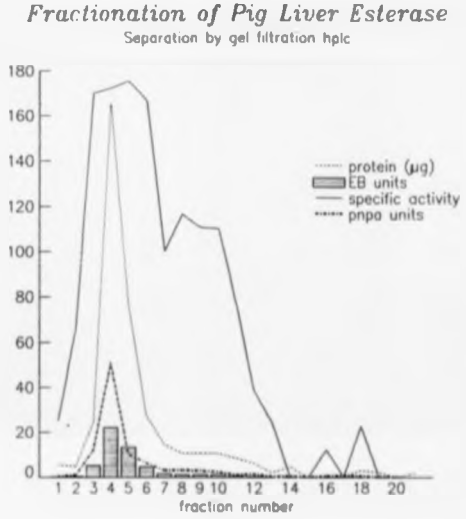


Figure 2.10

suggests that the preparation contains a single type of activity.

Levine *et al.* [80] carried out an extensive study of the kinetics of PLE dissociation using complement fixation. They arrived at rate constants for the steps involved based on a trimer dissociating. However, they did not check the molecular weight of

their oligomer. If, as shown in this work, it was not necessarily trimeric, this may make their rate constants invalid.

The dissociation study in this work was simply aimed at demonstrating the phenomenon, and not at quantifying it. The results are presented graphically in Figure 2.11 and typical chromatograms are shown in Figure 2.9. Figure 2.11 shows the ratio of oligomer to monomer on the y axis, the incubation pH on the x axis and the elution pH (the column pH) on the z axis. The immediate conclusion from this is that the state of association of PLE is highly correlated to pH. There is a breakpoint at pH 5.0, below which dissociation is rapid (within the timescale of the hplc analysis - 45 minutes). Above this point, there is a relatively smooth gradation, with greater association at higher pH. The isoelectric point (pI) of PLE lies between 5.5 and 4.9 (differing for the various isozymes). This suggests that, not surprisingly, surface charges are important in this reversible association.

Given that the association / dissociation of PLE is rapid, that both the monomer and oligomer are catalytically active and that there is no cooperativity, it is perhaps not surprising that the literature contains contradictory statements concerning the nature of PLE. Couple these features with the observations of monomer, dimer, trimer and hexamer, and it becomes clear that the degree of association of PLE is not important to its functioning or use as a biocatalyst, and that there may be no single, fixed state of oligomerization. This suggestion is supported by the findings of Bachiboo [83], who found that crude bovine liver esterase was [illegible]meric, and only associated during purification (as the

concentration increased). It may well be that at cellular concentrations, PLE too is monomeric, and that association has no physiological significance.

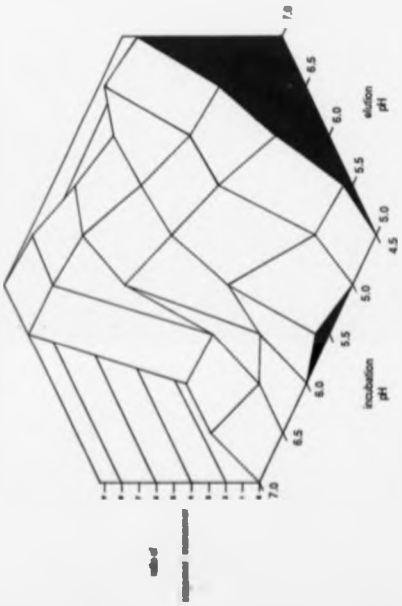


Figure 2.11 - The effect of pH on the association state of PLE

2.2.5 Ion - Exchange hplc

The peaks obtained using the DEAE - 5PW column were only partially resolved (chromatogram not shown). In addition, the shoulder on the second peak (340 mM NaCl) was approximately half the height of the parent peak. The degree of resolution suggested that complete separation of the components was unlikely on this type of column. The peaks could either be due to partial separation of isozymes, or of the various oligomers. Given the degree of difficulty encountered generally in separating the isozymes, it is thought unlikely that the peaks could be explained by separation of isozymes.

The first peak (325 mM NaCl) is very sharp, but both the second peak and its' shoulder (350 mM NaCl) are broad. From this, it is not inconceivable that the first peak is the monomer, and the others are oligomers. It is known that increasing concentrations of salts promote dissociation which, given the eluting NaCl gradient, could account for the broad character of these peaks.

2.2.6 Covalent Immobilization

PLE was immobilised onto two supports;

i) An aldehyde based polymer (Courtaulds plc, Coventry, U.K.) [84] and

ii) Eupergit C (Rohm Pharma, Darmstadt, FRG).

PLE has previously been immobilised onto Eupergit C with moderate retention of activity (48%) and good specific activity (1300 U/g support) [85]. While high immobilization yields were achieved in this work with Eupergit C (80 - 96 %, based on residual soluble activity), the immobilised activities were poor (20 - 36 %). Thus, approximately 60 % of the activity was repeatedly lost on immobilization. The specific activities obtained, at 700 U/g, were approximately half those obtained by Laumen *et al.* [85].

Immobilization onto the aldehyde polymer gave significantly higher immobilised activities (85 %), with only 1 % of the total activity appearing to be lost. The soluble residual activity, at 14 %, might have been decreased if the the reaction time had been extended. However, following the recommended procedure (P. M. Radley, personal communication) of 1 - 10 mg/ml protein and 0.1g polymer/ml led to low specific activities (29 U/g) when compared to those obtained with Eupergit C. Increasing the protein concentration in order to rectify this was not attempted. Other specific activities obtained with this support are 24 U/g for alkaline phosphatase and 450 U/g for β-galactosidase (P. M. Radley, personal communication).

Ligands are bound to Eupergit C by a reaction between

$-NH_2$ or -SH groups and an oxirane group on the polymer. Extraneous ammonium salts, amines and sulphydrides can compete with the protein for the available oxirane groups, and can significantly reduce the immobilised protein yield. However, this cannot account for the low immobilised activities experienced with Eupergit C in this work, as the immobilisation yields (based on residual soluble activity) were repeatedly high. Additionally, the three different techniques employed to remove ammonium sulphate prior to immobilisation all produced similar results. This suggests that the immobilisation process itself is responsible of the loss of activity.

2.3 Introduction to Experimental Sections

References are given to known compounds as they appear.

All solvents and starting materials were dried and / or purified prior to use according to the procedures described in "Purification of Laboratory Chemicals" (Perrin, Armarego and Perrin, 2nd ed., Pergamnon Press, 1985) unless otherwise stated.

Physical data is presented in the format suggested by the Royal Society of Chemistry.

Nuclear magnetic resonance spectra were recorded using the instruments listed below at the operating frequencies indicated.

Instrument	Frequency (MHz)		
nucleus	^{1}H	^{13}C	^{19}F
Bruker WH 400	400.13	100.62	-
Perkin Elmer R34	220.0	-	-
Bruker WH 90	90.0	-	84.67

Chemical shifts are quoted in ppm from tetramethylsilane as internal reference for proton and carbon-13 spectra, unless otherwise stated. Fluorine shifts are quoted in ppm from $CFCl_3$ as internal standard.

Mass spectra were recorded using a Kratos MS 80 spectrometer.

Infra - red spectra were recorded using a Perkin Elmer 580 - B spectrometer.

Optical rotations were recorded using an Optical Activity Ltd. AA - 1000 polarimeter. Rotations were measured at the sodium D line (589 nm) using 2 dm path length cells.

Melting points were recorded using a Gallenkamp digital melting point apparatus and are quoted uncorrected.

Autotitration and stationary pH (pHstat) experiments were performed using a Radiometer Copenhagen RTS 822 recording titration system.

Gas - liquid chromatographic analyses were performed using a Pye 104 gas chromatograph. Packed glass columns were used, which were six feet in length. The carrier gas was nitrogen.

High pressure liquid chromatography were performed using a Gilson isocratic system with a variable wavelength UV - Vis detector and a Rheodyne injector.

Thin layer chromatography was carried out using Merck Kieselgel F_{254} 0.2 mm precoated plates. Spot detection was by UV fluorescence quenching, potassium permanganate spray or phosphomolybdic acid / ethanol spray.

Flash chromatography [86] was performed using Merck Kieselgel 60 silica gel (230 - 400 mesh). Approximately 50 - 100 g silica gel was used per g of compound, depending on the degree of difficulty of separation. The solvent flow was assisted by compressed air, such that the solvent front moved at approximately 4 cm / minute.

Ether refers to diethyl ether.

Petrol refers to the petroleum fraction boiling in the range 40 - 60°C, unless otherwise stated.

2.4 Experimental for Chapter 2

2.4.1

Assay for Esterase Activity - Determination of Initial Rates

Standard Assay

Ethyl butyrate (distilled) was dissolved in phosphate buffer (pH 7.0 or 7.5, 67 mM) to give a final concentration of 12.5 mM.

Of this stock solution, 10 ml was equilibrated at 32°C with stirring. Pig liver esterase (typically 3.33 mg/ml, 130 U/mg, 50 µl) was added. The decreasing pH was maintained at 7.0 or 7.5 by the addition of NaOH (0.1 M) from an automatic titrator. The reaction was followed for 14 minutes.

One esterase unit = the amount of protein required to hydrolyse ethyl butyrate at the rate of one µmol/min. This is equivalent to the addition of 10 µl/ min alkali.

Determination of Initial Rates

The initial rates of hydrolysis of ethyl butyrate by pig liver esterase were determined using the assay method outlined above. Substrate concentration was varied between 0.51 and 10.17 mM.

[ethyl butyrate](mM)	v(µmol/min)
10.17	21.3
8.14	18.3
7.12	16.7
6.10	14.4

[ethyl butyrate](mM)	v(μmol/min)
5.09	12.8
4.07	12.5
3.05	8.9
2.03	8.8
1.02	7.0
0.51	5.5

The data was analysed by plotting it in the following transformations;

1, [s] v. v

2, The Lineweaver - Burk plot (1/[s] v. 1/v). This is biphasic. Both sections are linear and give the following kinetic constants;

10.17 < [s] > 3.05 V_{max} = 38.9 μmol/min

K_m = 10 mM

3.05 < [s] > 0.51 V_{max} = 10.4 μmol/min

K_m = 0.45 mM

3, The Eadie - Scatchard plot (v v. v/[s]). This is biphasic. Both sections are linear and give the following kinetic constants;

10.17 < [s] > 3.05 V_{max} = 48.9 μmol/min

K_m = 13.9 mM

3.05 < [s] > 0.51 V_{max} = 10.6 μmol/min

K_m = 0.48 mM

Does PLE Exhibit Cooperativity?

Using the data obtained above, a linear Hill plot (log [s] v. log (v/(V_{max} - v))) was constructed.

The V_{max} values obtained from the Lineweaver - Burk plot were used. If the V_{max} value used is that for the wrong section of data, then the Hill plot deviates seriously from linearity for those data points. Thus two data sets were constructed:

1, V_{max} = 38.9 μmol/min.

log [s]	log (v/(V_{max} -v))
0.911	-0.052
0.852	-0.217
0.785	-0.222
0.707	-0.310
0.610	-0.325
0.484	-0.527

n = 0.944

2, V_{max} = 10.4 μmol/min.

log [s]	log (v/(V_{max} - v))
0.484	0.775
0.307	0.740
0.0086	0.314
-0.292	0.0502

n = 1.003

2.4.2

The Effect of Water - Soluble Cosolvents on Pig Liver Esterase Activity

Pig liver esterase (10 mg/ml, 130 U/mg, 10 µl per assay) activity was assayed against ethyl butyrate using the pH - stat technique.

The assay solution contained ethyl butyrate (12.5 mM, from a stock solution), phosphate buffer (pH 7.5, 67 mM, from a stock solution) and cosolvent (see tables), to a total volume of 10 ml. This was stirred at 32°C. Pig liver esterase was added, and the uptake of NaOH (0.1 M) titrant was followed for 7 minutes.

Alcohols

alcohol(% v/v)	relative activity	methanol	ethanol
0		1	1
1		0.779	0.869
2.5		0.617	-
3		-	0.747
5		0.448	0.646
7.5		0.316	0.501
15		0.241	0.256

Ketones

ketone(% v/v)	relative activity	acetone	2-butanone
0		1	1
0.5		-	0.954
1		1.223	0.879
1.5		-	0.818
2		1.478	0.773
2.8		1.518	-
3		1.595	0.644
3.1		1.623	-
3.2		1.618	-
3.4		1.641	-
4		1.526	0.538
5		1.390	0.364
6		1.189	-
7		1.092	-
7.5		1.021	-
8		0.952	-
10		0.837	-
20		0.275	-
30		0.113	-

Synthesis of 2-chloroethyl butyrate

2-chloroethanol (8 g, 0.1 mol, anhydrous) was cooled to -70°C with stirring. Butyryl chloride (10.5 ml, 0.12 mol) was added dropwise over a period of 40 minutes. The mixture was brought to room temperature and stirred overnight. The mixture was washed with

aq.$NaHCO_3$ (1% w/v, 50 ml) and H_2O (50 ml), and dried ($MgSO_4$). The crude product was distilled bulb to bulb (110°C / 15 mm), to give 8.8 g (59 mmol) colourless oil, 2-chloroethyl butyrate.

Yield = 60%.

^1H NMR (220 MHz, $CDCl_3$ / TMS) : δ = 1.0 (t, 3H, CH_3); 1.7 (q, 2H, CH_2CH_3); 2.35 (t, 2H, CH_2CO_2); 3.7 (t, 2H, CH_2Cl); 4.35 (t, 2H, CH_2OC).

Comparison of the Inhibitory Effects of Product Alcohols on PLE Activity

Pig liver esterase (10 mg/ml, 130 U/mg, 3 µl) activity was assayed against methyl-, ethyl-, and 2-chloroethyl butyrate in the presence of methanol, ethanol and 2-chloroethanol respectively, by the assay method outlined in section 2.4.1.

Methyl butyrate / methanol

[methanol] (mM)	relative activity	% v/v
0	1	-
124	0.91, 0.87	0.5
247	0.85, 0.81	1
494	0.53, 0.51	2
741	0.47, 0.46	3
1235	0.33, 0.33	5
1853	0.26, 0.25	7.5
2470	0.21, 0.20	10

Ethyl butyrate / ethanol

[ethanol] (mM)	relative activity	% v/v
0	1	-
172	0.87	1
516	0.75	3
860	0.65	5
1290	0.50	7.5
2580	0.26	15

2-chloroethyl butyrate / 2-chloroethanol

[2-chloroethanol] (mM)	relative activity	% v/v
0	1	-
15	1.00, 0.98	0.1
30	0.96, 0.96	0.2
45	0.94, 0.93	0.3
60	0.88, 0.86	0.4
75	0.86, 0.82	0.5
112	0.83, 0.82	0.75
224	0.73, 0.71	1.5
298	0.62, 0.61	2.0
373	0.59, 0.55	2.5
447	0.56, 0.53	3.0
596	0.42, 0.41	4.0
745	0.35, 0.34	5.0
1175	0.20	7.9
1341	0.20	9.0

2.4.3

Eserine Inhibition of Pig Liver Esterase

Pig liver esterase (10 mg/ml, 130 U/mg, 10 µl) was incubated in the presence of eserine (physostigmine, 0 - 1 mM) for 3 minutes at room temperature, in phosphate buffer (pH 7.0, 67 mM).

The residual esterase activity was then assayed at pH 7.0 by the ethyl butyrate assay outlined in section 2.4.1.

[eserine] (µM)	relative activity
0	1
1	0.92, 0.94
5	0.91, 0.91
10	0.90, 0.89
50	0.90, 0.89
100	0.90, 0.90
500	0.84, 0.81
1000	0.79, 0.74

Phenylmethylsulphonyl Fluoride Inhibition of Pig Liver Esterase

Pig liver esterase (10 mg/ml, 130 U/mg, 10 µl) was incubated in the presence of phenylmethylsulphonyl fluoride (0 - 1 mM) for 30 minutes at room temperature, in phosphate buffer (pH 7.0, 67 mM).

The residual esterase activity was then assayed at pH 7.0 by the ethyl butyrate assay outlined in section 2.4.1.

[pmsf] (μM)	relative activity
0	1.0
1	0.99
5	1.0
10	0.98
50	0.88
100	0.66
500	0.35
1000	0.34

2.4.4

Estimation of the Molecular Weight of PLE by Gel Filtration hplc

A TSK G 3000 SW gel filtration hplc column was calibrated with the following proteins; cytochrome c (12.5 kd), chymotrypsinogen A (25 kd), hen egg albumin (45 kd), BSA (68 kd), aldolase (158 kd), catalase (240 kd) and ferritin (450 kd). The eluting solvent was phosphate buffer (pH 7.5, 67 mM) plus NaCl (10 mM), and the flow rate was 0.25 ml/min. Eluent was continuously monitored at 280 nm. The deadvolume was determined with dextran (254 nm).

Pig liver esterase was then analysed under the same conditions.

Sample	t_e (mins)	log mw (kd)
cytochrome c	60.4, 59.1	1.097
chymotrypsinogen A	50.8	1.398
hen egg albumin	41.8	1.653
(dimer)	36.4	1.954
BSA	38.5	1.833
(dimer)	33.2	2.134
aldolase	31.0	2.199
catalase	27.5	2.380
ferritin	26.2	2.653
dextran	25.6	>6
		Estimated mw (kd)
Pig liver esterase	33.6, 33.4	124.4, 122.6
	42.3, 42.1	55.7, 54.6
	57.9, 57.7	13.8, 13.8
	59.1, 59.0	<0.1

Gel Filtration hplc of Pig Liver Esterase and Fraction Characterisation

Pig liver esterase (2 mg/ml, 171 µl, 45.5 U) was fractionated by gel filtration hplc using a TSK G 3000 SW column. The eluting solvent was phosphate buffer (pH 7.0, 67 mM) with a flow rate of 0.5 ml/min. The eluant was continuously monitored at 280 nm. Twelve minutes after injection (dead volume of 6 ml), 24 0.5 ml fractions were collected. These were stored at 4°C.

Each fraction was subjected to three different assays:

1. Esterase activity was estimated using the ethyl butyrate

assay outlined in section 2.4.1.

2. Esterase activity was estimated using an assay based on the hydrolysis of p-nitrophenyl acetate to p-nitrophenol in phosphate buffer at pH 7.1 and at 32°C. The reaction was monitored at λ = 410 nm [81].

3. The protein concentration was estimated using the Coomassie Blue binding microassay [82]. A calibration curve was established using pig liver esterase at 2 - 20 µg.

Specific activity was calculated by dividing the ethyl butyrate units by the protein content (mg) of each fraction.

fraction	protein(µg)	units(ab)	sp. activity	p-nitrophenol(amol)
1	5.5	0.14	25.3	0.31
2	4.75	0.32	67.0	1.06
3	24.3	5.07	170.0	12.06
4	165.7	22.14	176.2	51.06
5	76.0	13.32	175.2	23.21
6	27.4	4.37	166.7	10.11
7	14.4	1.44	100.2	6.21
8	10.6	1.24	116.5	3.11
9	10.6	1.17	110.7	3.11
10	10.6	1.17	110.2	2.91
11	8.4	0.65	77.5	1.31
12	6.1	0.24	38.5	1.06
13	2.0	0.05	24.5	1.31
14	4.5	0.00	0.0	0.31

fraction	protein(μg)	units(eb)	sp. activity	p-nitrophenol(nmol)
15	1.0	0.00	0.0	0.41
16	1.0	0.01	12	0.21
17	0.5	0.0	0.0	0.41
18	3.0	0.07	22.7	0.81
19	2.0	0.00	0.0	0.41
20	0.0	0.00	0.0	0.06
21	1.75	0.00	0.0	0.21
22	0.0	0.00	0.0	0.26
23	0.0	0.00	0.0	0.06
24	0.0	0.00	0.0	0.00

The Effect of pH on the Monomer / Dimer Ratio of PLE

Pig liver esterase (2 mg/ml, 20 μl) was incubated in phosphate buffer (see table) for 10 minutes. The monomer / dimer distribution was then examined by gel filtration hplc using a TSK G 3000 SW hplc column. The eluting solvent was phosphate buffer (see table) at a flow rate of 0.5 ml/min. Eluent was continuously monitored at 280 nm. All buffers were 67 mM.

incubation pH	Elution pH	dimer:monomer
7.0	7.0	0.25:1
7.0	5.0	1.5:1
6.5	7.0	0.00:1
6.5	6.5	0.22:1
6.5	5.0	1.36:1
6.0	7.0	7.00:1
6.0	6.5	6.04:1

Incubation pH	Elution pH	oligomer:monomer
6.0	6.0	7.78:1
6.0	5.0	1.32:1
5.5	7.0	7.00:1
5.5	6.5	6.82:1
5.5	6.0	6.46:1
5.5	5.5	4.62:1
5.5	5.0	0.85:1
5.0	6.5	8.91:1
5.0	6.0	5.40:1
5.0	5.5	5.15:1
5.0	5.0	0.86:1
4.5	6.5	8.40:1
4.5	6.0	5.08:1
4.5	5.5	3.13:1
4.5	5.0	0.54:1

2.4.5

Analysis of PLE by Anion - Exchange hplc

Pig liver esterase (5 mg/ml, 10 µl) was analysed by anion - exchange hplc using a TSK DEAE - 5PW hplc column. The equilibrating solvent was phosphate buffer (pH 6.5, 67 mM). The eluting solvent was phosphate buffer (pH 6.5, 67 mM) plus NaCl (1.0 M). The eluent was continuously monitored at 280 nm. The flow rate was 0.5 ml/min and the gradient ran for one hour.

Components were eluted at 32.5 % (325 mM NaCl) and 34 % (340 mM NaCl). The second peak had a prominent shoulder at 35 % (350 mM NaCl).

2.4.6

Immobilization of PLE on Courtauld's Aldehyde - based Polymer

Pig liver esterase (10 mg/ml suspension, 150 µl, 195 U) was dissolved in phosphate buffer (pH 7.5, 1M, 75 ml) with stirring, to give a final concentration of 0.02 mg/ml. Courtauld's aldehyde - based immobilization polymer (6 g) was added and the mixture was stirred at room temperature for 4 hours. The polymer was filtered off, using a sinter funnel, and washed with H_2O (45 ml). The polymer was stored at 4°C.

The extent of immobilization was determined using the ethyl butyrate assay outlined in section 2.4.1.

Immobilised activity	165.4 units (84.8 %).
Soluble activity	28.0 units (14.4 %).
Unaccounted for	1.6 units (0.8 %).

Immobilised specific activity 29 U/g wet weight.

Immobilisation of PLE on Eupergit C

Pig liver esterase (10 mg/ml suspension, 130 U/mg) was treated in such a way as to remove ammonium sulphate;

1, Ultrafiltration using Centricon-10 microconcentrators (Amicon, mw cutoff 10 kd), or

2, Dialysis, or

3, Gel filtration using TSK G 3000 SW hplc columns.

All three techniques gave quantitative recovery of activity.

Buffer strengths and PLE concentrations were adjusted to 1 M (pH 7.5, phosphate) and 1.5 - 3 mg/ml respectively. Eupergit C (0.1 g/mg protein) was added and the mixture was stood at room temperature for 48 hours. The Eupergit C was collected by vacuum filtration, using a sinter funnel. The Eupergit C was washed three times with buffer, and stored in buffer at 4°C.

The extent of immobilisation was determined using the ethyl butyrate assay outlined in section 2.4.1.

Immobilised activity	21 - 36 %.
Soluble activity	4 - 20 %.
Unaccounted for	59 - 60 %.

Immobilised specific activity 700 U/g wet weight.

Chapter 3 - Biotransformations of *trans*-1,2-diacetoxycyclopentane

3.1 Introduction

As noted earlier (section 1.3.1), the hydrolysis of (±)-*trans*-1,2-diacetoxycyclopentane (10) by PLE leads to products of only moderate optical purity. Although the distribution of products is affected by the extent of hydrolysis, their enantiomeric excesses do not significantly vary (see Figure 3.1). This lack of specificity could have several origins. It is known that the isozymes constituting PLE have different substrate, inhibitor, cosolvent and kinetic characteristics. From this, it is not inconceivable that the enantiospecificities might also vary, and that this, further complicated by differing catalysis rates amongst the isozymes, could be responsible for the lack of specificity with certain substrates. This argument could also be used to account for occasional erratic results when different batches of enzyme are used.

Two strategies can be adopted when testing the validity or otherwise of this hypothesis. One involves the separation and testing of the isozymal fractions for specificity against a range of substrates. Such a study has been carried out [87]. The other strategy uses specific inhibitors with the "mixed isozyme" system. Eserine and phenylmethylsulphonyl fluoride (pmsf) have been shown to be both potent and highly specific inhibitors of the two most easily

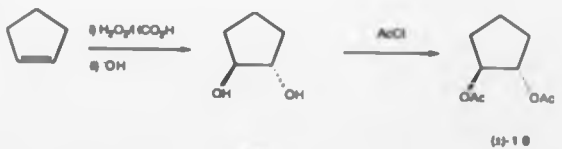
(±)- **10**	**10**	**16**	**4**
% hydrolysis	%ee	%ee	%ee
25	54	50	-
63	-	50	50
74	-	63	47

Figure 3.1 - PLE catalysed hydrolysis of (±)-trans-1,2-diacetoxycyclopentane (10) [33]

separated isozymes of PLE [67]. If enantiospecificity varies across the spectrum of isozymes, then it should be evident with these two populations.

The use of these inhibitors allows one to selectively remove a single activity from the mixture. Should varying specificities be detected and found to be a problem, then the use of these inhibitors would provide an experimentally simple solution. The inhibitor pmsf has previously been used in this context, to inhibit serine proteases during a biotransformation catalysed by a crude preparation of pig pancreatic lipase [63].

While the PLE catalysed hydrolysis of 10 poses an interesting enzymological question, it does not provide a practical route to optically pure chiral cyclopentanoids. *Pseudomonas fluorescens* lipase (PFL) has been shown to be a highly specific catalyst for effecting this reaction [51,88a].



31%

(±)-10

74%

Figure 3.2 - Synthesis of (±)-*trans*-1,2-diacetoxycyclopentane (10)

3.2 Results and Discussion

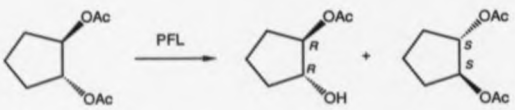
3.2.1 Synthesis of *trans*-1,2-diacetoxycyclopentane (10)

The racemic title compound, 10, was synthesized in two steps from cyclopentene *via* the racemic *trans*-1,2-dihydroxycyclopentane (4) as illustrated in Figure 3.2. The yields were 31% and 74% respectively, giving an overall yield of 23%. The products were characterized by ^{1}H NMR, which were satisfactory.

3.2.2 PPL Catalysed Hydrolysis of 10

The procedure outlined by Xie *et al.* [53,88a] for the resolution of 10 was repeated. The results obtained (see Figure 3.3) are in close agreement with those reported. The procedure was not optimized, which may account for the slightly lower chemical yields obtained in this work (36% and 37% for 10 and 10 respectively, compared to 43% and 42% [53]). The specific rotation of 10 (+21.1°) is close to the reported value of +22.3° (for e.e. >99%), suggesting that 10 was obtained in high optical purity. Xie *et al.* do not give a rotation for the recovered diacetate. The value obtained here (-20.1°) is of a similar magnitude to that for 10, again suggesting high optical purity.

The absolute configuration of (+)-10 is reported to be (1*S*,2*S*). This assignment was based on comparison with the known (*S*,*S*)-(-)-dihydroxycyclopentane and on the c.d. spectra of the corresponding bis(*p*-methoxy)benzoates [88a].



(±)- **10** **16** $[\alpha]_D = +21.1^\circ$ **10** $[\alpha]_D = -20.1^\circ$

Lit. $+22.3^\circ$ [88a]

Figure 3.3 - PFL catalysed hydrolysis of (±)-*trans*-1,2-diacetoxycyclopentane (**10**)

3.2.3 Inhibited PLE Catalysed Hydrolysis of 10

The hydrolysis of **10** by PLE was investigated under three sets of conditions. For one, a control experiment, PLE was not subjected to any incubations prior to the hydrolysis. For each of the other two, PLE was incubated in the presence of an isozyme specific inhibitor prior to the hydrolysis (see section 2.2.3 and [67]). To ensure continued inhibition during the reaction, the inhibitors were also present in the reaction media. The normalised reaction rate profiles are depicted in Figure 3.4.

In the selective hydrolysis of a racemic diester, the reaction could cease at one of three possible endpoints. The product distribution for these endpoints (all of 0.5 equi-eq.'n) , in an ideal situation are;

i) diester, mono-ester (endpoint of 0.5 mol-eq.),

ii) diester, diol/di-acid (endpoint of 1.0 mol-eq.) and

iii) mono-ester, diol/di-acid (endpoint of 1.5 mol-eq.).

Although there are small rate changes at these points for the control experiment, overall the rate profile does not significantly deviate from that for a standard first order reaction. The consequence of this is the only moderate enantiomeric excesses quoted in Figure 3.1. It can clearly be seen that a similar situation pertains to the two isozyme inhibited hydrolyses.

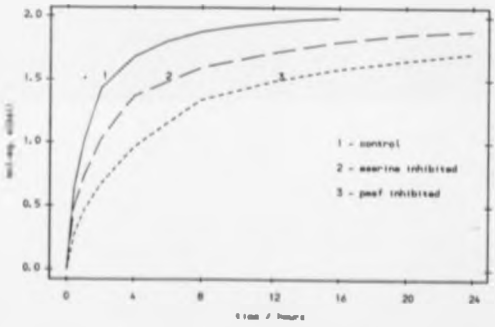


Figure 3.4 - Inhibited PLE catalysed hydrolysis of (±)-*trans*-1,2-diacetoxycyclopentane (18)

The hydrolyses catalysed by the inhibited PLE preparations were slower than that for the control experiment. In the time required to complete this hydrolysis, the eserine inhibited reaction has reached 1.8 mol-eq.'s, and pmsf, 1.6 mol-eq.'s. At the inhibitor concentrations used, eserine and pmsf inhibited PLE preparations display 77% and 34% residual activity respectively against ethyl butyrate (see section 2.2.3). The residual activities against 10 and 16 are higher in both cases. This could suggest either incomplete isozyme inhibition, or just reflect the inherent difference in activities towards the various substrates for the non - inhibited isozymes. Given the length of the incubation with the inhibitors prior to the hydrolysis and the inclusion of the inhibitors in the reaction media, it is thought unlikely that incomplete isozyme inhibition occurred.

The lack of clear breakpoints or even rate changes for the inhibited reactions implies that both 10 and 16 are suitable substrates for all the isozymes constituting PLE. This suggests that the isozymes probably display broadly similar stereochemical requirements; that low and moderate e.e.'s are inherent features of the system, rather than being due to competing species with grossly different stereospecificities. These findings are supported by a parallel study [88], in which the isozymes were fractionated. Of the substrates analysed, two were hydrolysed stereospecifically by "whole" PLE. In these cases, all the fractions were found to be highly specific. The other substrates tested were hydrolysed by PLE with specificities ranging from poor to good. These specificities were likewise mirrored closely, but with some variation, by all of

the isozymal fractions.

Several groups have reported that the stereochemical outcome of a PLE catalysed hydrolysis can be advantageously altered by the addition of cosolvents, especially DMSO [89,90]. Guanti *et al.* [90] conjectured that the isozymes may respond differently to the added cosolvent, thus bringing about the observed changes. Given what is now known about the specificities of the isozymes, this seems unlikely, as it implies that the isozymes will display significantly different stereospecificities.

3.3 Experimental for Chapter 3

3.3.1

trans-1,2-dihydroxycyclopentane (A)

Cyclopentene (20 g, 0.29 mol) was added dropwise to a mixture of formic acid (170 ml, 98%) and hydrogen peroxide (30 ml, 30%) with stirring at 0°C. The reaction mixture was stirred for a further 2 hours at 0°C, and then for 18 hours at room temperature. Formic acid was removed *in vacuo* at 45°C. The residue was cooled to 0°C, and NaOH (12.5 M, 52 ml) was added over a period of several hours, ensuring that the temperature remained below 40°C. The mixture was taken up in a minimum volume of H_2O and extracted with ethyl acetate (5 x 100 ml). The aqueous layer was saturated with NaCl and the extraction repeated (3 x 100 ml). The extracts were pooled, dried ($MgSO_4$), and the solvent removed *in vacuo*, giving a yellow oil. The crude product was distilled bulb to bulb

(85°C / 0.1 mm, Lit. 92-93°C / 2 mm [88b]), giving a colourless oil (9.0 g, 88 mmol).

Yield = 31%.

IR (film) : ν 3,380 (br, -OH), 2,945 (w, CH), 2,870 (w, CH), 1,080-70 cm^{-1} (m, doublet, C-O).

^1H NMR (220 MHz, $CDCl_3$ / TMS) : δ = 1.55 (m, 2H, CH_2CHOH); 1.75 (m, 2H, CH_2CHOH); 2.05 (m, 2H, $CH_2(CH_2)_2$); 3.55 (br s, 2H, OH); 4.00 (dt, 2H, $(CHOH)_2$).

trans-1,2-diacetoxycyclopentane (10)

(±)-trans-1,2-dihydroxycyclopentane (6.0 g, 58 mmol) was dissolved in anhydrous pyridine (11.5 ml) containing 4-dimethyl aminopyridine (35 mg) with stirring at 0°C. Acetyl chloride (11.0 g, 140 mmol, freshly distilled) was added dropwise over 1 hour. The reaction mixture was stirred overnight. The mixture was taken up into ether (75 ml) and washed with 1% $NaHCO_3$ (2 x 50 ml). The aqueous washings were pooled and back - extracted with ether (2 x 75 ml). The ethereal components were pooled, dried ($MgSO_4$) and the solvent removed *in vacuo*, giving 8.4 g (78%) crude product. This was distilled bulb to bulb (75°C / 0.1 mm), giving a colourless oil (8.0 g, 43 mmol).

Yield = 74%.

^1H NMR (220 MHz, $CDCl_3$ / TMS) : δ = 1.65 (m, 2H, CH_2CHO); 1.75 (m, 2H, CH_2CHO); 2.05 (s, 6H, $(CH_3CO_2)_2$); 2.08 (m, 2H, $CH_2(CH_2)_2$); 5.10 (dt, 2H, $(CHOCO)_2$).

3.3.2

Pseudomonas fluorescens Lipase Hydrolysis of 10

(±)-*trans*-1,2-diacetoxycyclopentane (102 mg, 0.55 mmol) was dissolved in phosphate buffer (pH 7.0, 67 mM, 5 ml) with stirring at 32°C. *Pseudomonas fluorescens* lipase (Amano lipase P, 50 mg, 1,500 U) was added. The decreasing pH was maintained at 7.00 by the addition of NaOH (0.1 M) from an automatic titrator. After the addition of 0.5 mol - equivalents of alkali (20 hours), the reaction mixture was continuously extracted with ether (50 ml, 24 hours). The ethereal layer was dried ($MgSO_4$) and the solvent removed *in vacuo*, giving a mixture of diacetate and monoacetate. These were separated by flash chromatography using ether - petrol (1:1) as eluent. This gave (-)-*trans*-1,2-diacetoxycyclopentane (38 mg, 37%) and (+)-*trans*-1-acetoxy-2-hydroxycyclopentane (28 mg, 36%).

(+)-*trans*-1-acetoxy-2-hydroxycyclopentane (16)

Yield = 36%.

$[\alpha]_D^{20}$ = +21.1° (c = 1.4, $CHCl_3$). Lit. [88a] +22.3°.

^1H NMR (220 MHz, $CDCl_3$ / TMS) : δ = 1.75 (m, 4H, $(CH_2CO)_2$); 2.05 (m, 2H, $CH_2(CH_2)_2$); 2.10 (s, 3H, CH_3CO_2); 2.95 (br s, 1H, OH); 4.15 (m, 1H, CHOH); 4.85 (m, 1H, CHOCO).

(-)-*trans*-1,2-diacetoxycyclopentane (18)

Yield = 37%.

$[\alpha]_D^{20}$ = -20.1° (c = 1.4, $CHCl_3$).

^{1}H NMR : data identical to that for the racemate (see section 3.3.1).

3.3.3

Pig Liver Esterase Hydrolysis of 18

(±)-*trans*-1,2-diacetoxycyclopentane (38 mg, 0.2 mmol) was dissolved in phosphate buffer (pH 7.0, 67 mM, 2 ml) containing either i) no inhibitors (control), ii) eserine (physostigmine, 1 mM) or iii) p-toluenesulphonyl fluoride (1 mM) with stirring at 32°C. Pig liver esterase (65 U) was incubated in phosphate buffer (pH 7.0, 67 mM, 500 µl) containing either i) no inhibitors, ii) eserine (1 mM) or iii) p-toluenesulphonyl fluoride (1 mM) at room temperature for one hour, and was then added to the substrate solution. The decreasing pH was maintained at 7.00 by the addition of NaOH (0.1 M) from an automatic titrator. The reactions were not worked - up.

Run	i	ii	iii
	(control)	(eserine)	(pmsf)
time/hrs	eq. alkali	eq. alkali	eq. alkali
0	0.0	0.0	0.0
0.4	0.32	0.494	0.269
0.8	0.902	0.638	0.404
1	1.022	0.74	0.471
2	1.431	1.025	0.673
4	1.68	1.37	0.962
6	1.804	-	-
8	1.88	1.597	1.341
10	1.924	-	-
12	1.956	1.715	1.49
14	1.978	-	-
16	1.99	1.802	1.586
18	2.0	-	-
20	-	1.86	1.654
22	-	1.888	-
24	-	-	1.712
26	-	1.942	-
32	-	2.0	-

Chapter 4 - PLE Catalysed Hydrolysis of Diethyl Penta-2,3-dienedioate

4.1 Introduction

Allenes are nonplanar molecules, with substituents on the allenic skeleton lying in two perpendicular planes. Consequently allenes may be chiral by virtue of an appropriate arrangement of achiral substituents on the allenic backbone. In this situation, only two (or more) different substituents are required to produce "axial chirality". In the standard monocentric system ("asymmetric atom"), four different substituents are required.

Axially chiral allenes may have two, three or four different substituents, as depicted in Figure 4.1.

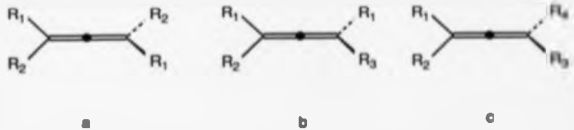


Figure 4.1 - Disposition of substituents for axially chiral allenes

Assignment of configurations for these systems is related to the Cahn - Ingold - Prelog convention. The enantiomer is viewed along the allenic axis, with front groups taking precedence over rear

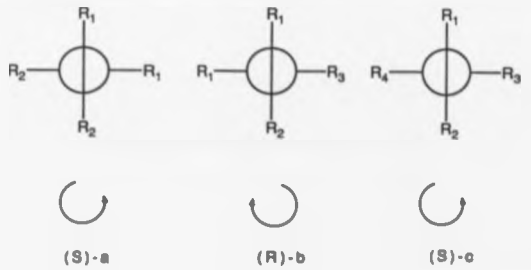


Figure 4.2 - Assignment of configurations for axially chiral allenes

groups. Accordingly, the structures in Figure 4.1 have the configurations illustrated in Figure 4.2.

The assignment of configuration is a central topic of stereochemistry. Two standard techniques for achieving this end are X-ray diffraction and correlation with closely related known compounds. In the case of allenes, a variety of other techniques, including o.d., optical rotatory dispersion, and theoretical calculations of both the sign and magnitude of the rotation at the sodium D line (589 nm) have proved extremely reliable.

Methods of resolving carboxylic allenes are still based largely on classical fractional crystallization methods with alkaloids such as brucine, strychnine, quinine and cinchonidine. The allene penta-2,3-dicarboxylate (17), which is closely related to the structures in this work, has been partially resolved by fractional

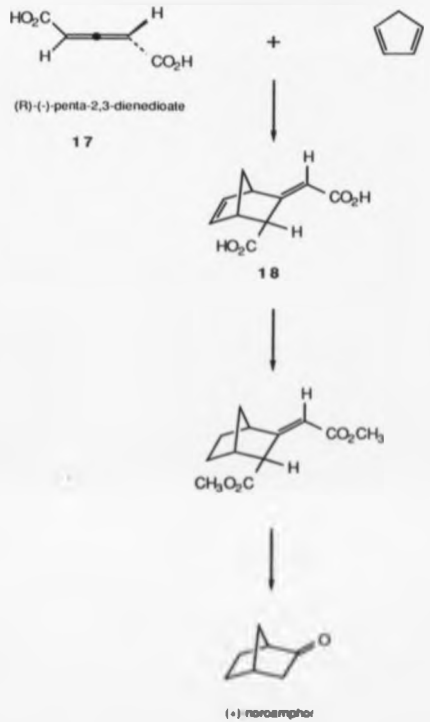


Figure 4.3 - Determination of the absolute configuration of 17 [91,92]

crystallization as the quinine salt [91,92]. The absolute configuration of (-)-17 was determined by an ingenious method. The partially resolved acid was treated with cyclopentadiene, giving two isomers, of which 18 was the major isomer (see Figure 4.3). The minor product (not shown) was due to the (+)-17 present. 18 was isolated and transformed into (+)-norcamphor of known configuration. From this procedure it was concluded that the major enantiomer in the partially resolved mixture was (S)-(-)-17. It was thought that the lower energy required for the approach of the cyclopentadiene from the side of the "out of plane" hydrogen, rather than from the side of the "out of plane" carbonyl group was the controlling factor for this *cis* addition [92].

An empirical rule has been proposed by Lowe [93] and extended by Brewster [94], which correlates the sign of the rotation at the sodium *D* line with the absolute configuration. This rule relates the rotation sign to the average polarizabilities of the allenic substituents in a manner similar to the Cahn - Ingold - Prelog convention. The rule is highly successful for disubstituted systems, such as 17, but is less so for more highly substituted allenes [95].

The Lowe - Brewster rule has been largely superseded by a semi - empirical theory due to Ruch and Schonhofer [96-99]. This theory, based on "chirality functions" for the allenic ligands, predicts both the sign and magnitude of the specific rotation. Hence, a single measurement could give both the configuration and optical purity of a sample. This rule is restricted to acyclic allenes with achiral ligands.

Generally it is assumed that direct physical methods (spectral and chromatographic) are more reliable than theoretical rules for the determination of optical purities. Accordingly, there have been a number of studies of the use of lanthanide shift reagents and chiral solvating reagents with allenes [100-106]. The chiral solvating reagent, α-naphthylethylamine, is not able to resolve the enantiomeric ^{1}H NMR spectra of a range of allenic alcohols, ketones and phosphoryl derivatives [100]. The shift reagent, europium (D-3-heptafluorobutyrylcamphorate)$_3$, with CCl_4 or 1,1,2-trichloro-1,2,2-trifluoroethane as solvent, has been shown to be able to resolve the enantiomers of a number of allenic alcohols, esters and alkylallenic carboxylic acids [101,104,105], including dimethyl penta-2,3-dienedioate.

Primary allenic alcohols (of type a in Figure 4.1) can be partially resolved by esterification with lauric acid in hexane catalysed by the lipase from *Candida cylindracea* [106]. The optical purities obtained were moderate, being higher for the more highly hindered alcohols and for reactions terminated well before (for the ester) or well after (for the alcohol) 50% conversion.

A rather curious biotransformation is the isomerisation of dec-3-ynoyl-N-acetylcysteamine (19) to deca-2,3-dienoyl-N-acetylcysteamine (Figure 4.4) [107]. This PLE catalysed reaction proceeds *via* a prototropic rearrangement, and does not inactivate the enzyme.

Ramaswamy *et al.* [30] have shown that PLE can discriminate between the enantiomers of a range of alkylallenic esters of types b and c (Figure 4.1). An example of this is the

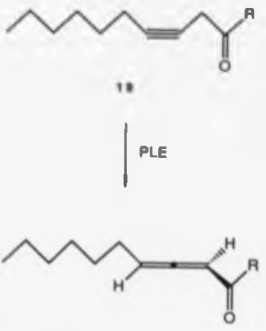


Figure 4.4 - PLE catalysed isomerisation of 19 [107]

hydrolysis of 1 (Figure 1.5). As with the work of Gil *et al.* [106], it was found that the stereospecificity improved with the "bulkiness" of the substrate.

Biotransformations of allenic esters of type a (Figure 4.1) have not appeared in the literature. Substrates of this type are, by definition, at least diesters. In some respects, they are analogous to typical PLE diester substrates. They therefore are attractive substrates for PLE mediated resolutions.

4.2 Results and Discussion

4.2.1 Synthesis of Diethyl Penta-2,3-dienedioate

Racemic diethyl penta-2,3-dienedioate (21) was synthesized using the procedure of Bryson and Dolak [108a], with modifications. The major alteration was the use of anhydrous ethanol in the second step, rather than methanol. This was to prevent the formation of the dimethyl ester *via* transesterification. Dimethoxyethane (DME) was substituted for tetrahydrofuran (THF) in the final step. The yield of diethyl-3-chloro-2-pentenedioate (20) was 45%, and of 21, 77%. This gave an overall yield of 35%. Both products were characterized by ^{1}H NMR, which were satisfactory.

4.2.2 Proton NMR Resolution of 21

Using the chiral shift reagent europium (D-3-heptafluorobutyrylcamphorate)$_3$ (Eu(hfbc)$_3$), it was possible to distinguish between the enantiomers of 21 by ^{1}H NMR. This resolution was observed with $CDCl_3$ or $CCl_2F.CClF_2$ as solvent, but not with CCl_4. The peak resolution was typically 50 - 60% (by area), with magnetic field strength and temperature having little effect. Europium (D-3-trifluoroacetylcamphorate)$_3$ in $CDCl_3$ caused severe line broadening, and no separation was observed.

These findings are largely in agreement with those of Lang and Hansen [104], who studied the dimethyl ester. The main

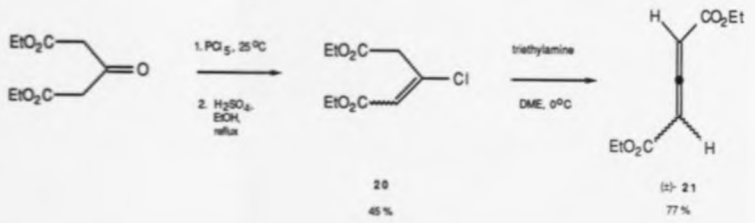


Figure 4.5 - Synthesis of (±)-diethyl-2,3-pentadienedioate (21) [108]

difference being that they observed resolution with CCl_4 as solvent using $Eu(hfbc)_3$. They do not mention the use of $CDCl_3$.

Because of the paramagnetic nature of europium, increasing magnetic field strengths leads to line broadening. Therefore, the lowest field strength able to produce the desired resolution was used (90 MHz). The resolution of an NMR spectrum can be considerably increased if the data is collected with the spectrometer "locked" onto a resonant frequency. In common with most FT - NMR spectrometers, the machine used in this work (a Bruker WH 90) has a deuterium lock channel. This precluded the use of $CCl_2F.CClF_2$ as a solvent for "locked" spectra, unless an external lock was used. This is experimentally complex and is generally avoided if at all possible. The discovery that $CDCl_3$ was an equally effective solvent circumvented this problem. Its use allowed high quality spectra to be obtained routinely at 90 MHz, whereas obtaining similar quality spectra at 220 MHz or with $CCl_2F.CClF_2$ (at either frequency) was problematic.

More complex spectra than those of Lang and Hansen [104] were obtained because of the use of the ethyl, rather than the methyl ester. However, this was not a problem at 90 MHz, because the linewidths were sufficiently narrow to allow all peaks to be located easily.

4.2.3 Hydrolysis Studies

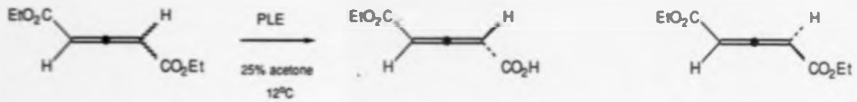
Diethyl penta-2,3-dienedioate (21) is only sparingly soluble in water. When a polypropylene reaction vessel was used, this led to the substrate "clinging" to the hydrophobic walls of the reaction vessel, and not mixing thoroughly with the aqueous phase. This resulted in a slow reaction (as monitored by alkali uptake), and the decomposition of the substrate. No allenic product was found. This could either be due to decomposition of the hydrolysis product or because no carboxylic allene was formed. The tendency of 21 to cling to the walls of the reaction vessel was reduced by the use of a glass vessel. However, the same result was obtained.

The standard technique for bringing insoluble substrates into contact with PLE is by the use of cosolvents (see section 2.2.2). Both DMSO and acetone have previously been used successfully in this context. In this work both were used at initial concentrations of 25% v/v. At this level, DMSO precipitates phosphate buffer salts, and so tris - HCl buffer was used. With DMSO, there was a marked change in the rate of alkali uptake at 0.5 mol-eq. (by a factor of 13). This implies considerable specificity. Both the neutral and acidic extracts were largely DMSO. However, they also contained allenic resonances in their ^{1}H NMR and had measurable optical rotations. Because the concentrations of the allenes in the extracts were not known, it was not possible to calculate specific rotations. It was decided not to distil the extracts because of the lability of the allenic structure and the possibility of racemising any optically enriched / pure components.

Therefore counter - current extraction with H_2O/CH_2Cl_2 was used to partition the allenes from the DMSO. Unfortunately, this led to the decomposition of the allenes.

With acetone as cosolvent, the reaction temperature was reduced to 12°C to reduce the rate of acetone loss by evaporation. This temperature was found to be a suitable compromise between cosolvent loss and reaction rate. At 4°C, the reaction rate was negligible (<0.25 mol-eq. in 7 days). The rate change at 0.5 mol-eq. was less marked than with DMSO. Upon work - up, the reason for this became apparent - the reaction had reached only 0.3 mol-eq. The recovery of unhydrolysed 21 and the product, (-)-ethyl penta-2,3-dienedioate (22) was quantitative. The e.e. of (+)-21 was easily established as 15 ±3 % using the method outlined in section 4.3.2. This method failed with 22, producing severe line broadening. However, an e.e. of 35 ±7 % can be estimated using the value for 21 with the extent of hydrolysis.

By correlation with (*S*)-(-)-17, the absolute configurations are (*S*)-(+)-21 and (*S*)-(-)-22. These assignments are in agreement with both the Lowe - Brewster [93,94] and Ruch - Schonhofer [96-98] rules. The latter rule also predicts specific rotations of +272° and -254° for (+)-21 and (-)-22 respectively. The experimental rotation for 22 (-75.1°) suggests an e.e. of 30%. This is within the error for the value calculated previously. An extremely small positive rotation was obtained for (+)-21, suggesting an e.e. of only 0.3 % (i.e. racemic). In view of the fact that the e.e. was determined directly and independently by ^{1}H NMR, this value can be disregarded.



(±)-**21**

(R)[1]-(-)-**22**

$[\alpha]_D$ = -75.1°

ee = 30.3%[2]

ee = 35±7%[3]

(S)[1]-(+)-**21**

$[\alpha]_D$ = +0.8°

ee = 0.3%[2]

ee = 15±3%[3]

Figure 4.6 - PLE hydrolysis of 21 in 25% aqueous acetone

1 - By comparison with (R)-(-)-pentadienedioate (17) and in agreement with the Lowe - Brewster rules [93,94].

2 - Calculated using the rules of Ruch and Schonhofer [96-99].

3 - Calculated from the ^{1}H NMR of (+)-21 with europium (D-3-heptafluorbutyrylcamphorate)$_3$ and the yields.

The outcome of the hydrolysis of this substrate parallels the findings of Ramaswamy *et al.* [30] for less highly substituted allenic esters. This suggests that the replacement of the proton ligands with more bulky groups such as phenyl or cyclohexyl will be necessary to obtain stereoselective PLE hydrolyses of allenes of type a.

4.3 Experimental for Chapter 4

4.3.1

Diethyl-3-chloro-2-pentenedioate (20)

Diethyl acetone-1,3-dicarboxylate (55.5 g, 0.27 mol, freshly distilled) was stirred at 20°C under N_2. Phosphorus pentachloride (62 g, 0.3 mol) was added portionwise over 2 hours, ensuring that the reaction temperature remained below 40°C. The mixture was warmed to 40°C for 90 minutes, and then cooled in ice. Crushed ice (100 ml) was added and stirring was continued at 0°C for 30 minutes. The aqueous mixture was extracted with CH_2Cl_2 (3 x 100 ml). The organic layers were pooled, dried ($MgSO_4$) and the solvent removed *in vacuo*. This gave a crude red product (46.0 g, R_f 0.5, ether - petrol 1:1), which was used without further purification. The crude oil was dissolved in anhydrous ethanol (300 ml) and sulphuric acid (20 ml, 98%) was added carefully. The reaction mixture was boiled gently under reflux for 24 hours. After cooling, the mixture was poured into H_2O (100 ml) and saturated with NaCl. This was extracted with ether (3 x 100 ml). The organic layers were washed

successively with aq.$NaHCO_3$ (100 ml, sat.) and aq.NaCl (100 ml, sat.), then dried ($MgSO_4$) and the solvent was removed *in vacuo*. This gave 36.0 g crude oil. This was distilled bulb to bulb (85°C / 0.1 mm, Lit. 50-60°C / 0.02 mm for the dimethyl ester [108a]), giving 20.8 g (0.12 mol) colourless oil, diethyl-3-chloro-2-pentenedioate.

Yield = 45%.

R_f = 0.75 (ether - petrol, 1:1).

^1H NMR (220 MHz, $CDCl_3$ / TMS) : δ = 1.30 (t, 6H, J = 6.0 Hz, CH_3); 4.12 (s, 2H, CH_2CCl); 4.20 (m, 4H, CH_2CH_3); 6.30 (s, 1H, CHCCl).

Diethyl penta-2,3-dienedioate (21)

Diethyl-3-chloro-2-pentenedioate (17.6 g, 80 mmol) was dissolved in anhydrous dimethoxyethane (DME, 65 ml) with stirring at 0°C under N_2. Triethylamine (freshly distilled, 15 ml) was added dropwise over 30 minutes. The N_2 inlet was replaced with a drying tube, and the reaction stirred for 24 hours. The precipitate was removed by suction filtration. The precipitate was washed with ether (3 x 50 ml). The combined filtrates were washed successively with HCl (0.5 M, 3 x 50 ml) and aq.NaCl (50 ml, sat.). The ethereal layer was dried ($MgSO_4$) and the solvent was removed *in vacuo*, giving 10.0 g crude red oil. This was distilled bulb to bulb (90°C / 0.1 mm, Lit. 90°C / 0.2 mm for the dimethyl ester [108a]), to give 11.34 g (61.8 mmol) colourless oil, (o)-diethyl penta-2,3-dienedioate, which was stored under N_2 at -20°C.

Yield = 77%.

R_f = 0.6 (ether - petrol, 1:1).

^{1}H NMR (220 MHz, $CDCl_3$ / TMS) : δ = 1.30 (t, 6H, J = 7.0 Hz, CH_3); 4.25 (q, 4H, J = 7.0 Hz, CH_2); 6.05 (s, 2H, CH=C).

4.3.2

Attempted Resolution of the Enantiomers of 21 by Proton NMR

(±)-Diethyl-2,3-pentadienedioate (18.5 mg, 0.1 mmol) was dissolved in anhydrous $CDCl_3$ (0.6 ml) and its ^{1}H NMR spectrum (220 MHz) was recorded.

Europium (D-3-trifluoroacetylcamphorate)$_3$ was added in 0.1 mol - equivalent aliquots. The ^{1}H NMR spectrum was rerecorded after each addition. At 0.1 mol - equivalents, severe line broadening obscured all peak splitting. By 0.4 mol - equivalents, peaks were not discernible from baseline noise.

Resolution of the Enantiomers of 21 by Proton NMR

(±)-Diethyl-2,3-pentadienedioate (18.5 mg, 0.1 mmol) was dissolved in an anhydrous solvent (see table, 0.6 ml) and its ^{1}H NMR spectrum recorded either at 90 MHz or 220 MHz.

Europium (D-3-heptafluorobutyrylcamphorate)$_3$ was added in 0.1 - 0.2 mol - equivalent aliquots. The ^{1}H NMR spectrum was rerecorded after each addition.

Solvent	Frequency/MHz	T/°C	peak	resolution*	mol-eq.
CCl_4	220	20	all	0	0.1-0.6
$CDCl_3$	220	20	CH=	0.5	0.5
			CH_2	0.54	0.5
		20-50	CH_3	0.56	0.3
	90	20	CH_2	0.5	0.5
			CH_3	0.5	0.5
CCl_2FCClF_2	220	20	CH=	0.8	0.25
			CH_3	0.6	0.25

* by area

4.3.3

Attempted Hydrolysis of 21 by Pig Liver Esterase in a Wholly Aqueous Medium

(±)-Diethyl-2,3-pentadienedioate (36 mg, 196 μmol) was mixed with phosphate buffer (pH 7.0, 67 mM, 10 ml) in a polypropylene titration vessel with vigorous stirring at 32°C. Pig liver esterase (650 U, in 0.5 ml phosphate buffer) was added. The slowly decreasing pH was maintained at 7.00 by the addition of KOH (0.1 M) from an automatic titrator. After the consumption of 0.50 mol - equivalents of alkali (22 hours), the reaction mixture was extracted with ether (4 × 25 ml). The ethereal extracts were pooled, dried ($MgSO_4$) and the solvent removed *in vacuo*. The oil obtained contained no allenic resonances by ^1H NMR. The aqueous layer was acidified to pH < 2 (HCl), and the extraction repeated. This also gave no allenic resonances by ^1H NMR.

Attempted Hydrolysis of 21 by Pig Liver Esterase with DMSO Cosolvent

(+)-Diethyl-2,3-pentadienedioate (0.55 g, 3.0 mmol) was dissolved in DMSO (5 ml, distilled). This was added slowly to tris - HCl buffer (pH 7.5, 0.3 M, 15 ml) in a glass titration vessel with stirring at 25°C. A uniform white emulsion formed. Pig liver esterase (390 U, in 0.3 ml tris - HCl buffer) was added. The decreasing pH was maintained at 7.50 by the addition of NaOH (0.1 M) from an automatic titrator. After the consumption of 0.52 mol - equivalents of alkali (15 hours), the reaction was stopped. The ratio of the rates prior to and post 0.5 mol - equivalents was 13:1. The reaction mixture was extracted with CH_2Cl_2 (4 x 25 ml), acidified (HCl) to pH < 2, and re - extracted. Both the neutral and acidic extracts were dried ($MgSO_4$) and the solvent removed *in vacuo*. Both residues contained large amounts of DMSO (by ^1H NMR). Both residues were subjected to counter - current extraction using CH_2Cl_2 / H_2O in 10 tubes to partition the DMSO. This successfully removed the DMSO from the organic phases in tubes 1 - 6. However, the organic residues from both extractions no - longer contained allenic resonances (by ^1H NMR).

Hydrolysis of 21 by Pig Liver Esterase with Acetone Cosolvent at 12°C

(+)-Diethyl-2,3-pentadienedioate (188 mg, 1.0 mmol, freshly distilled) was dissolved in acetone (analar, 10 ml). This was added slowly to phosphate buffer (pH 7.0, 67 mM, 30 ml) in a

glass titration vessel with stirring at 12°C. Thermostating at 12°C was achieved by means of a thermocirculator attached to a running cold water tap. The solution remained clear. Pig liver esterase (650 U, in 0.5 ml phosphate buffer) was added. The decreasing pH was maintained at 7.00 by the addition of NaOH (0.45 N) from an automatic titrator. After the consumption of 0.56 mol - equivalents of alkali (14 hours), the reaction had stopped. The reaction mixture was extracted with CH_2Cl_2 (2 x 40 ml), acidified (HCl) to pH < 2, and re - extracted. Both the neutral and acidic extracts were dried ($MgSO_4$) and the solvent removed *in vacuo*. This gave unhydrolysed diester (128 mg, 0.70 mmol) from the neutral extract, and hydrolysed (-)-ethyl-2,3-pentadienedioate (45.7 mg, 0.29 mmol) from the acidic extract. By the use of the chiral shift reagent, europium (D-3-heptafluorobutyrylcamphorate)$_3$, the ee of the unhydrolysed diester was determined to be 15 ± 3%. The estimated ee of the hydrolysed mono - ester is 35 ± 7%, using the above data.

(+)-Diethyl penta-2,3-dienedioate (21)

Yield = 69%.

R_f = 0.6 (ether - petrol, 1:1).

$[\alpha]_D^{20}$ = +0.8° (c = 3.4, $CHCl_3$).

^1H NMR - data identical to that for the racemic material.

(-)-Ethyl penta-2,3-dienoate (22)

Yield = 29%.

R_f = 0.23 (ether - petrol, 1:1).

$[\alpha]_D^{20}$ = -75.1° (c = 0.75, $CHCl_3$).

^1H NMR (220 MHz, $CDCl_3$ / TMS) : δ = 1.30 (t, 3H, J = 6.7 Hz, CH_3); 4.25 (q, 2H, J = 6.7Hz, $CH_3\underline{CH_2}O_2CCH$); 6.10 (dd, 2H, J = 14.0, 6.6 Hz, CH=C=CH); 9.50 (br s, 1H, CO_2H).

^{13}C NMR (100.6 MHz, $CDCl_3$ / $CDCl_3$ = 77.0 ppm) : δ = 13.98 (CH_3); 61.77 (CH_2); 91.90 ($\underline{C}HCO_2H$); 92.73 ($CHCO_2\underline{C}$); 163.01 ($\underline{C}O_2CH_2$); 168.50 (CO_2H); 220.37 (=C=).

MS (EI) : m/z = 156 (M^+), 112.

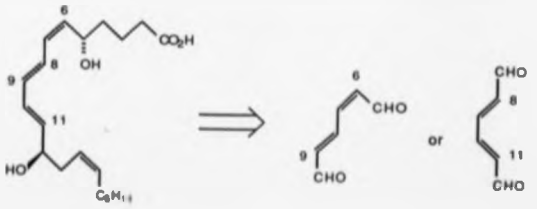
MS (CI, NH_3) : m/z = 174, 156.

Chapter 5 - Chiral Cyclobutene Derivatives from Biotransformations

5.1 Introduction

5.1.1 Chemical Aspects

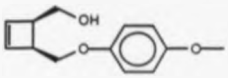
Interest in lipoxygenase - derived metabolites of arachidonic acid, such as leukotrienes [109] and lipoxins [110], has led to the development of synthetic routes to these classes of molecules. One approach is based on the use of hexa-2,4-dienals as central fragments, as illustrated in the retrosynthetic analysis of leukotriene B_4 [111].



leukotriene B_4

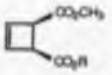
Figure 5.1 - Retrosynthetic analysis for leukotriene B_4 [111]

Suitable dienals can be prepared *via* stereoselective electrocyclic ring opening [112] (and isomerizations) of 1,2-disubstituted -3-cyclobutenes such as 23.



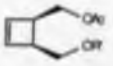
23

In turn, such *cis*-1,2-disubstituted-3-cyclobutenes can be prepared from the corresponding dicarboxylic acid anhydride [113]. The *meso*-dimethyl ester (24) can be prepared from the anhydride in 90% yield. The *meso*-diacetate (25) can be prepared by reduction of the anhydride with lithium aluminium hydride in ether, giving the *meso*-dimethanol (92% yield) [114], followed by acetylation. Both of these molecules are attractive substrates for esterase or lipase catalysed hydrolyses to the corresponding mono - ester (26) or acetate (27).



24 R = CH_3

26 R = H



25 R' = Ac

27 R' = H

Although thermal conrotatory electrocyclic ring opening is possible with the hydrolysis reaction products, a previous study on such a *cis*-3-cyclobutene-1,2-dicarboxylic acid mono - ester has shown that little selectivity between the two "allowed" products (2E,4Z and 2E,4E) can be achieved for this type of system [115]. That cyclobutenes similar to 27 can be ring - opened with considerable selectivity has been amply demonstrated with 29 [111].

5.1.2 Biotransformation Aspects

Considerable attention has been focused in recent years on biotransformations of cyclic *meso*-1,2- diesters, diacetates and dimethanol diacetates [22,34-38,53,55,60,61,116,117]. Much of this work has been discussed in earlier sections: 1.3.1 (pig liver esterase), 1.4.2 (*Pseudomonas fluorescens* lipase) and 1.4.3 (pig pancreatic lipase).

The highly stereoselective hydrolyses of *meso*-cycloalkane (n = 3-6) 1,2-dimethyl dicarboxylates catalysed by pig liver esterase (PLE) are illustrated in Figure 5.2. There is an interesting parallel between these hydrolyses and those of *trans*-cycloalkane-1,2-diacetates, also catalysed by PLE (Figure 1.6). In both series, there is a change in the configuration of the product as the ring size is increased. In both cases, this occurs at the cyclopentane stage.

There is a disagreement over the sign of the rotation of PLE generated methyl hydrogen *cis*-3-cyclobutene-1,2-dicarboxylate

(-)-(1R,2S)
>97% e.e.

31

(-)-(1R,2S)* - 28
>97% e.e.

(+)-(1S,2R)
17% e.e

32

(+)-(1S,2R)
97% e.e

Figure 5.2 - PLE Catalysed Hydrolysis of *meso* cycloalkane diesters [116,117]

* (+)-(1R,2S) according to Schneider *et al* [116]

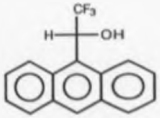
(28) within the literature. Sabbioni and Jones [117] state that it is laevorotatory, while Schneider *et al.* [116] obtained a positive rotation for the monoester of the same configuration. Not withstanding this, they agree on the absolute configuration of PLE generated **28**, which they determined by different methods.

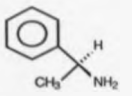
5.2 Results and Discussion

5.2.1 Proton NMR Resolution of the Enantiomers of the Hydrolysis Products

With any work on the stereochemistry of a system, it is important to be able to determine the relative proportions of the constitutive enantiomers. Whilst measuring optical rotations is experimentally simple, it is only of use if the specific rotation of the molecule in question is known. Additionally, measurements are sensitive to achiral and especially chiral impurities. Direct physical methods, whether chromatographic or spectroscopic, are much less prone to this problem. Such methods are usually based on the formation of diastereomeric compounds or complexes.

The chiral solvating reagent (*S*)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol (**29**) [118] has been used to determine the enantiomeric ratios of a range of alcohols, alkenes, amines and lactones. It was possible to distinguish between the enantiomeric ^{1}H NMR spectra of both methyl hydrogen *cis*-3-cyclobutene-1,2-dicarboxylate (**26**) and *cis*-3-cyclobutene-1,2-dimethanol





29

(S)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol

30

(S)-(-)-α-methylbenzylamine

monoacetate (27). In the latter case, all of the proton resonances were resolved. The singlet due to the acetyl group (δ = 2.0 ppm) gave rise to two singlets, separated by 2 Hz in the presence of 29 (see Figure 5.3). Although the singlet due to the methyl ester of 26 was also split in the presence of 29, the resolution was only 74% (by area) and the separation 1.3 Hz.

α-Methylbenzylamine (30) readily forms diastereomeric salts with chiral carboxylic acids. In this case, (-)-(S)-30 was used to distinguish between the enantiomers of 26 in $CDCl_3$ (see Figure 5.4). The salt did not precipitate. The signals due to the methyl ester were separated by 8.6 Hz, with baseline resolution. However, these peaks were not used when determining the enantiomeric excess because of the presence of other resonances with similar chemical shifts. These made identification of the minor component difficult. Instead, the signals due to the alkenic protons (δ = 5.80 ppm) were used. These two sets of doublets of doublets were well separated and easily quantified. The two outer signals are due to one diastereomer, and the central pair, the other (Figure 5.4b).

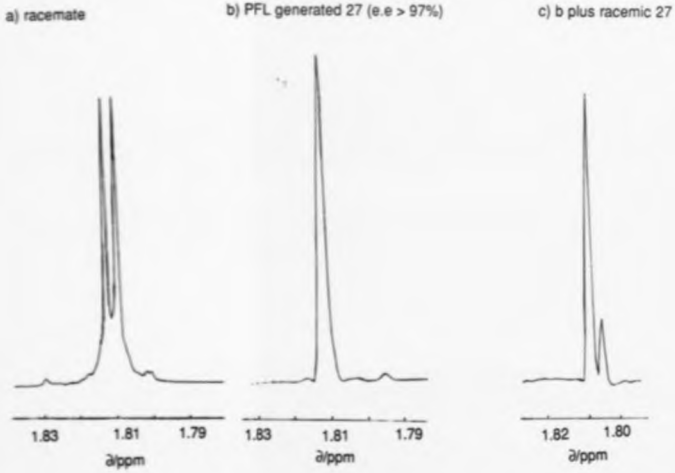


Figure 5.3 - Resolution of the ^{1}H NMR acetate signal of **27** in the presence of **29**.

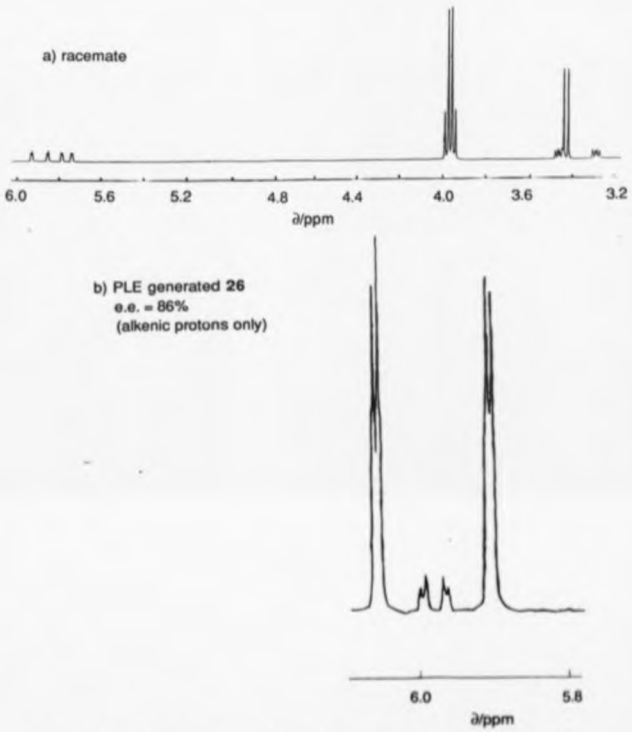


Figure 5.4 - Resolution of the ^{1}H NMR signals of **26** in the presence of **30**.

5.2.2 PLE Catalysed Hydrolysis of 24

The PLE catalysed hydrolysis of dimethyl *meso*-3-cyclobutene-1,2-dicarboxylate (24) is both rapid and highly specific. Under the reaction conditions employed, PLE displays 3.6% of the activity it displays against ethyl butyrate. This is a similar value to those for other cyclic *meso* diesters [117]. The form of the catalyst, whether free or immobilised, does not affect either the yield or stereochemical outcome of the reaction. The chemical yield is virtually quantitative (96%). The enantiomeric excess, at 86%, is high (see Figures 5.4b and 5.5). At this level, a single recrystallisation is usually sufficient to render material optically pure. However, unlike the racemic material, optically enriched half ester, 25, is not crystalline. Compound 25 was fully characterised (see section 5.3.2).

As this work was nearing completion, a near identical procedure was patented by Lukas [119]. The only significant difference from this work was that the pH of the hydrolysis reaction was 8.0. The chemical yields are nearly identical. The optical purity was determined in the patent using (*S*)-(+)-2B. No figure was given for the e.e.. Instead, it was stated that "*das optischen Antipoden wurde nicht festgestellt*" (the opposite optical form (enantiomer) was not detected). Lukas [119] based this statement on the methyl singlets. As mentioned previously, there is cluttering of peaks in this region of the spectrum. It is possible that he was simply unable to identify the minor component. No mention was made of the olefinic resonances. The signs of the optical rotations are

24

(+)-26
e.e = 86%
yield = 96%

Figure 5.5 - PLE catalysed hydrolysis of 24

the same in both pieces of work, but their magnitudes differ. This is probably a reflection of the different solvents used for the measurements.

Lukas concluded that he had obtained the (1*S*,2*R*) enantiomer. This assignment was based on the catalytic hydrogenation of (+)-**26**, giving (+)-**28** (see Figure 5.2). The absolute configuration of PLE generated **28** has, as previously noted, been established as (1*R*,2*S*) [116,117]. However, the rotation sign of (1*R*,2*S*)-**28** is disputed. Lukas based his assignment on the negative rotation of Schneider *et al.* [116], believing that he had the opposite enantiomer. The "consistently" positive rotation obtained by Sabbioni and Jones [117] appeared in print after the work of Lukas and suggests that his (1*S*,2*R*) assignment may be wrong. The more recent publication contains full experimental detail, in contrast to that of Schneider *et al.*. On this basis, it is thought that the newer, fuller data is more reliable, and that the configuration is in fact (1*R*,2*S*).

The new assignment suggests that PLE attacks the *pro-S*

centre of both 24 and its' saturated analogue, 31. The PLE-catalysed hydrolysis of 8 (an analogue of 24) and 32 yields the (1S,2R) enantiomers. Thus the presence of a double bond in the ring, in itself, is not sufficient to induce the reversal of stereospecificity for a given ring size that is required by the Lukas assignment.

The evidence for the (+)-(1R,2S)-26 assignment is strong, but not overwhelming. It is recognised that a definitive assignment cannot be made on this data alone, and that an unambiguous assignment is still required.

5.2.3 Benzylamide Derivatives (+)-26

Amides can be prepared by reacting a free carboxylic acid with 1,1'-carbonyldiimidazole (CDI) in a 1:1 molar ratio at room temperature [120]. An equimolar amount of amine is added after CO_2 evolution has ceased. The imidazole by - product can be removed by filtration and yields are usually high. When used in peptide synthesis, the mild conditions of CDI mediated reactions have been found to produce only minimal racemization (0.5%) [121].

When CDI was used in the synthesis of the benzylamide derivative of (+)-26, it was clear from both the ^{1}H NMR and tlc of the crude product that there were two products present. From the ^{1}H NMR spectrum of the imidazolamide intermediate (33), it was apparent that two intermediates were present, and that their ratios varied with time. Initially it was thought that the unexpected

product might be resulting from ring - opening. However, careful examination of the ^{1}H NMR spectrum of the mixture showed that no hexa-2,4-diene - like species were present. The second product was tentatively assigned the *trans* configuration. Formation of the second product could be minimised, but not eliminated, by the addition of benzylamine as soon as CO_2 evolution had subsided. Simultaneous addition of CDI and benzylamine to the reaction mixture failed to produce a reaction. This was presumably because of the formation of the benzylamine - 26 salt in preference to 33. The epimerisation of 33 was essentially complete in 30 minutes. The *cis*:*trans* ratio was then 1:8. The addition of further CDI did not alter this ratio.

The *trans* assignment of the unexpected product was confirmed from its' ^{1}H NMR coupling constants after purification. The optical rotation of the *trans* - benzylamide (34) (-255°) was significantly different from that of the *cis* - benzylamide (35) (+11.7°). Because of its cyclic nature, any epimerisation of the intermediate would change the configuration at that centre. While the NMR coupling and optical rotation data support the hypothesis of a clean epimerisation at C_2, the evidence was not conclusive. Circular dichroism (c.d.) spectra were recorded for (-)-34 and (+)-35 (see Figure 3.6). These suggest that C_1 has the same configuration in both molecules, while C_2 does not. This evidence proves that the epimerisation inverts the C_2 centre without affecting C_1. Therefore, starting from (+)-(1*R*,2*R*)-26, the absolute configurations would be (-)-(1*R*,2*R*)-34 and (+)-(1*R*,2*S*)-35. This situation is summarised in Figure 3.7.

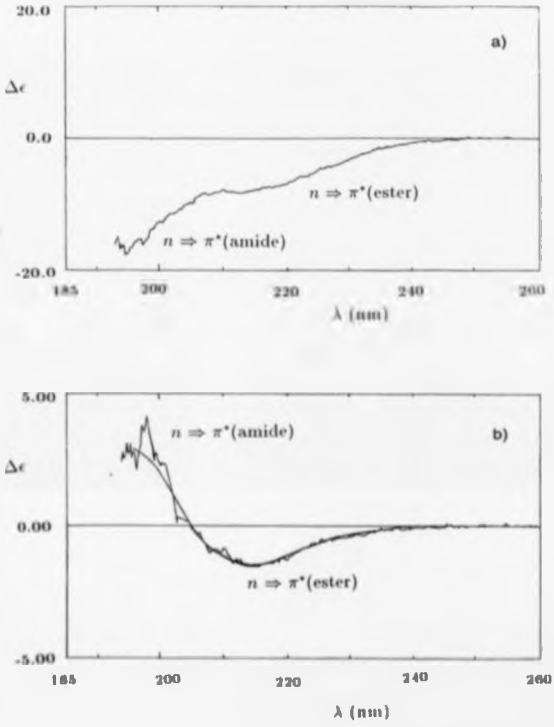


Figure 5.8 - c.d. spectra of a) 3.4 and b) 3.5

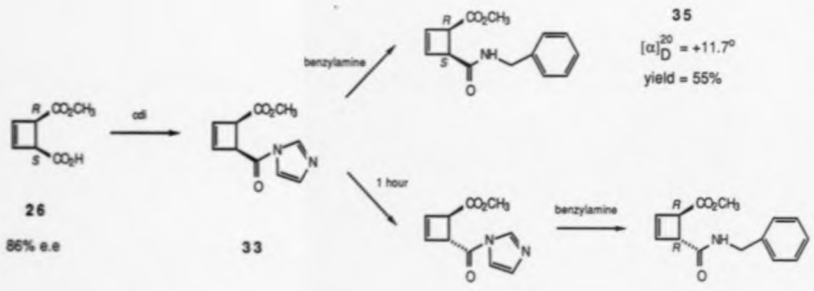


Figure 5.7 - Epimerization and Derivatization of (+)-26

The C_1 and C_2 substituents of 35 are nearly coplanar. This means that they will not meet the requirements of the Harada - Nakanishi exciton chirality method [122] for assigning absolute configurations. This problem does not apply to 34. However, the methylene group adjacent to the phenyl group makes the distance for charge transfer between the amide and phenyl groups too great. This makes 34 also unsuitable for c.d. configurational assignment.

The mechanism of epimerization presumably involves abstraction of the proton at C_2. Steric repulsion between the methyl ester and the bulky imidazole will then favour the trans epimer when the "anion" is quenched. It seems plausible that the proton is abstracted by a nitrogen of the imidazole ring. This could be either intramolecularly (possibly as depicted in Figure 5.8) or intermolecularly by the free imidazole. As the epimerization occurs subsequently to the formation of 33, it is clear that the proton abstraction is not by the imidazole leaving group (on the formation

Figure 5.8 - Possible intramolecular mechanism of epimerization of 33

of the intermediate). Such *cis:trans* epimerizations have been observed previously with *meso*-1,2-disubstituted-3-cyclobutenes, such as 1,2-dimethyl-3-cyclobutene [123]. The *cis* and *trans* epimers of this compound, on thermally - induced ring opening, cleanly isomerized to (2E,4Z)- and (2E,4E)-hexadiene respectively. Proton abstraction at C_1 and C_2 has been demonstrated for **24** (see figure 5.9) [124]. The dianion **36** is stable at room temperature and has been spectroscopically characterized. Chemical and spectroscopic data suggest that the anion is not stabilized by delocalisation.

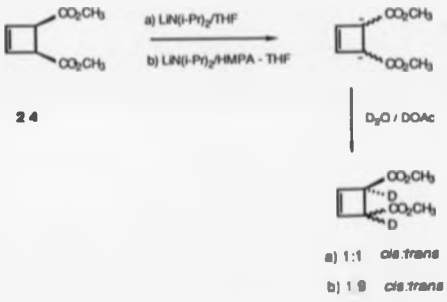


Figure 5.9 - Proton abstraction at C_1 and C_2 of 24 [124]

3.2.4 Screening of Hydrolytic Enzymes for Selectivity Against the Diacetate 25

A total of twelve enzymes were screened for selectivity in the hydrolysis of *meso*-3-cyclobutene-1,2-dimethanol diacetate (25). Of these, only α-chymotrypsin was unable to catalyse the hydrolysis of the substrate. Of the remaining eleven enzymes, evidence of little or no specificity was found for the lipases from wheatgerm, *Aspergillus niger*, *Candida cylindracea* and Lipozyme (immobilised *Mucor sp.* lipase), and for PLE, electric eel acetylcholinesterase and phospholipase A_2. The lipases from *Pseudomonas fluorescens* (PFL), *Rhizopus javanicus* (RJL) and pig pancreas (PPL) displayed differing degrees of specificity. RJL was the only enzyme tested to produce (-)-27 with significant stereoselectivity. However, varying the reaction conditions (e.g. the use of a water - immiscible cosolvent) did not increase the stereoselectivity. It is thought that replacement of the acetate groups with a longer C-chain acid (e.g. valerate) would be necessary to increase the specificity of the RJL catalysed hydrolysis.

The hydrolysis of the corresponding saturated analogues (Figure 1.10) by PLE leads to essentially racemic monoacetates [61,117]. However, PLE is specific towards the cyclohexene analogue (Figure 1.7) [34].

The lack of significant stereoselectivity displayed by PPL with substrate 25 was a surprise, especially given the consistently high (1*R*,2*S*) stereoselectivity it displays with the saturated analogues (Figure 1.10) [60,61]. Additionally, Hammerle

and Gais [62] obtained an e.e. of 86% (57% yield) for 27 using crude PPL (steapsin) in water. No experimental detail was given for their procedure, and the result could not be reproduced.

5.2.3 Further Investigations of the PPL Catalysed Hydrolysis of the Diacetate 25

Because of the unexpected result obtained with PPL and 25, further experiments were instigated. As previously discussed (section 1.4.4), the crude PPL preparations used for biotransformations contain only low levels of lipases. Hammerle and Gais [62] and Tombo *et al.* [64] have shown that the enantiospecificity decreases and reaction times lengthen when purified PPL is used as the catalyst. Three preparations of PPL with differing lipase activities were examined to see if this was a general phenomenon, or specific to the substrates they examined. Since only low selectivity was observed with the crude preparation, only the rate was examined. It is clear from this study, that the higher the specific lipase activity, the lower the hydrolysis rate, unit for unit. This is in agreement with the earlier work, and suggests that the enantioselectivity of the reaction will not be improved by using purer PPL preparations. A high molecular weight fraction has been isolated from crude PPL, which shows e.e. enhancements of 15 - 40% over the crude mixture [64]. However, even a 40% e.e. enhancement of the poor 8% e.e. would not give a synthetically useful result. Because this fraction is not readily

available to practitioners of biotransformations, it was not considered a useful avenue to explore.

A significant feature of lipases is their catalytic activity at interfaces. This allows lipases to hydrolyse water - insoluble substrates, and significantly different outcomes can be achieved for hydrolysis reactions by altering the phase characteristics of the system [50,51]. There are two methods currently used to produce multiphase reactions with substrates exhibiting some water solubility. One is the use of low polarity [52] water - immiscible cosolvents. The other is the use of a high ionic strength buffer to reduce the substrate solubility (salting - out). This leads to the formation of substrate micelles. The addition of NaCl (0.4 M) was found to be sufficient to achieve this with diacetate **25**. Carrying out the hydrolysis in this manner doubled the e.e.. However, an e.e. of 16% was not considered adequate to justify further work, especially given the high specificity of PPL for the same product.

Further details of the procedure of Mazzarle and Gais [62] are now available (H.-J. Gais, personal communication; Gais and Mazzarle, J. Am. Chem. Soc., manuscript in preparation). It appears that the use of diisopropylether (15% v/v) as a cosolvent is "*absolutely necessary*" for stereospecificity. The procedure suffers from a high catalyst:substrate ratio (5:1 w/w). Much of the non - protein material present in crude PPL is soluble in organic solvents, making chromatography essential. A further complication of crude PPL - catalysed hydrolyses of cyclic *meso*-1,2- dimethanol diacetates is reproducibility. Adam *et al.* [123] report that they

have not always been able to obtain the e.e.'s of Kasel *et al.* [60] for the hydrolyses depicted in Figure 1.10, which are analogues of 27.

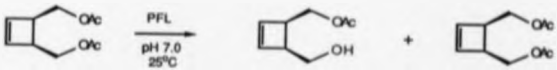
5.2.6 PPL Catalysed Hydrolysis of the Diacetate 25

As noted in section 5.2.4, *Pseudomonas fluorescens* lipase is highly stereoselective in the hydrolysis of 25, producing (+)-27 with an e.e. of 86% at one mol-equivalent hydrolysis. This procedure was optimised (see Figure 5.10). The monoacetate can be prepared optically pure by halting the hydrolysis at 0.8 mol-equivalents. The minor enantiomer could not be detected by the method outlined in section 5.2.1 until racemic material was added (see Figure 5.3b and c). The accuracy of the method is estimated to be ±3%. Therefore, the optical purity of (+)-27 using the optimised procedure is >97%. The extraction solvent was changed to dichloromethane, which gave quantitative recovery of (+)-27 and 25 (99.8%). Continuous extraction was not used, thus avoiding the possibilities of thermally - induced ring opening or acetyl migration (racemisation). The cyclobutenes (+)-27 and 25 were purified by flash chromatography. Because of the *meso* structure of 25, it could be recycled without further manipulation. The yield of (+)-27 after chromatography was 75% (94% with respect to 27 only). Monoacetate (+)-27 was fully characterised (see section 5.3.6).

It is not possible to predict the absolute configuration

of (+)-27 from literature precedents of PFL catalysed hydrolyses alone. As discussed in section 1.4.2, this is because PFL preferentially hydrolyses acetates of the *R* configuration, but there is one known exception. The exception, meso-1,2-diacetoxy-cyclopentane (9) is the closest analogue of 25 previously specifically hydrolysed with this lipase. No hydrolyses of cyclic meso-1,2-dimethanol diacetates using PFL have been published.

Although of only low specificity in this work, it was found that PFL catalysed the formation of the same enantiomer of 27 as PFL (based on the optical rotation and the NMR chiral analysis method). Gais and Hemmerle (*op. cit.*) also obtained the (+)- enantiomer. They determined the absolute configuration to be (1*R*,2*S*) by catalytic hydrogenation of (+)-27, giving (-)-37. The absolute configuration of (-)-37 has been determined previously by conversion to a known lactone [14,60]. This assignment suggests that this reaction is the second example of an *S* - centre specific acetate hydrolysis catalysed by PFL.



25 (+)-27 25
e.e > 97% yield = 16%
yield = 75%

Figure 5.10 -*Pseudomonas fluorescens* lipase catalysed hydrolysis of 25

5.2.7 Derivatisation of (+)-27

Protecting groups were sought for the primary hydroxyl group of 27 which would simultaneously give crystalline adducts and would be removable under mild conditions. It was intended to analyse the structure *via* single crystal X-ray diffraction and determine the absolute configuration *via* anomalous dispersion methods. Three derivatives were investigated (see Figure 5.11). These were [126,127]:

(-)-*cis*-2-[(4-phenyl)benzoyloxymethyl]-3-cyclobutene-1-methanol acetate (38),

(+)-*cis*-2-[(4-toluenesulphonyl)oxymethyl]-3-cyclobutene-1-methanol acetate (39), and

cis-4-[(9-phenylxanthen-9-yl)oxymethyl]-3-cyclobutene-1-methanol acetate (40).

Of these, only (-)-38 was isolated as an analytically pure solid. Derivative (+)-39 was an oil, which decomposed on standing back to 27 (optical purity not determined) and 4-toluenesulphonic (tosic) acid within 7 days. This behaviour was unexpected as tosyl derivatives are typically stable and highly crystalline. Compound 40 was characterised by ^{1}H and ^{13}C NMR, but could not be purified. The crude product was crystalline, but recrystallisation, chromatography and washing all failed to remove excess 9-chloro- / 9-hydroxy- 9-phenylxanthene.

The 4-phenylbenzoate group has been used as a hydroxyl protecting group in prostaglandin synthesis [128], yielding easily

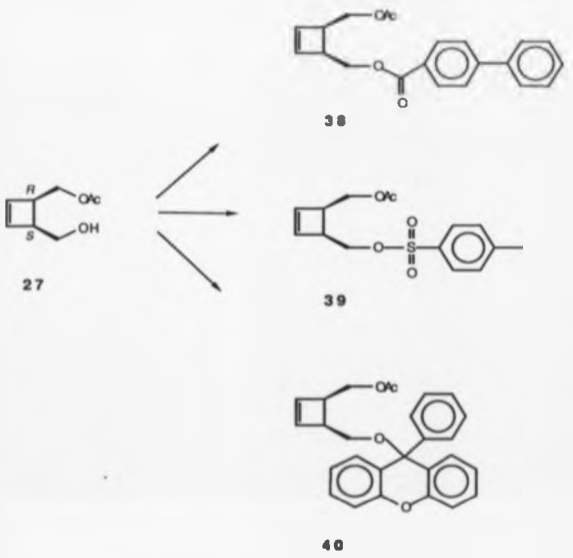


Figure 5.11 - Derivatization of (+)-27

handled, characterized and purified intermediates. The crystallinity of (-)-38 proved to be low, the product appearing more like a precipitated solid than a crystalline substance. This rendered the derivative unsuitable for characterization by standard X-ray techniques. The n.d. spectrum of (-)-38 was recorded (see Figure

5.12). The possibility of charge transfer between the ring double bond and the 4-phenylbenzoate group existed. This would have allowed an unambiguous absolute configurational assignment to be made. Unfortunately, the lack of any Cotton - effects [122] showed that no charge transfer was occurring. However, there is no evidence for any change in configuration during the derivatisation, and so the configuration is probably (1*R*,2*S*), as depicted in Figure 5.11.

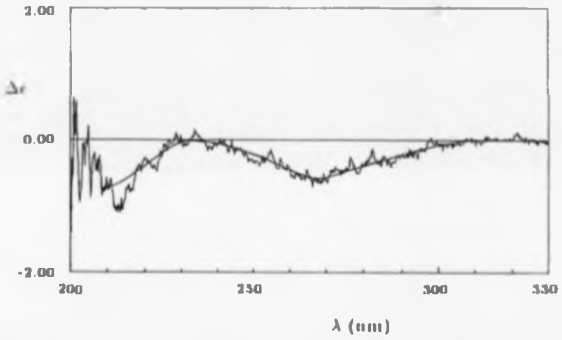


Figure 5.12 - c.d. spectrum of 3 8

5.3 Experimental for Chapter 5

5.3.1

Analysis of the Optical Purity of Methyl Hydrogen *cis*-3-cyclobutene-1,2-dicarboxylate (26)

Procedure 1

Methyl hydrogen (±)-*cis*-3-cyclobutene-1,2-dicarboxylate (26) (4.0 mg, 26 μmol) was dissolved in anhydrous CCl_4 (0.6 ml) containing C_6D_6 (10%) for locking. The ^1H NMR spectrum (400 MHz) was recorded.

The chiral solvating reagent (S)-(+)-2,2,2-trifluoro-1-(9-anthryl) ethanol (21.5 mg, 78 μmol) was added, and the spectrum was rerecorded.

δ	m	plus csr	δ	m	comments
3.66	s		3.44	d	Δ = 1.32 Hz, 74% resolved
3.92	s		3.59	dd	
6.25	dd		6.15	ddd	overlapping

Procedure 2

Methyl hydrogen (±)-*cis*-3-cyclobutene-1,2-dicarboxylate (26) (3.6 mg, 23 μmol) was dissolved in anhydrous $CDCl_3$ (0.6 ml). The ^1H NMR spectrum (400 MHz) was recorded.

The chiral solvating reagent (S)-(-)-α-methylbenzylamine (6.0 mg, 50 μmol) was added, and the spectrum was rerecorded (see Figure 5.4a).

δ	m	plus car	δ	m	comments
3.66	s		3.43	2s	baseline resolved, Δ = 8.6 Hz
3.92	s		3.28	dd	Δ = 0.18 ppm
			3.46	dd	
6.25	dd		5.80	2dd	baseline resolution

When samples were determined to be non - racemic (ee > 10%), racemic material was added, and the spectrum was rerecorded.

Analysis of the Optical Purity of *cis*-3-cyclobutene-1,2-dimethanol monoacetate (27)

(±)-*cis*-3-Cyclobutene-1,2-dimethanol monoacetate (27) (2mg, 13 μmol) was dissolved in anhydrous CCl_4 (0.6 ml) containing C_6D_6 (10%) for locking. The ^1H NMR spectrum (400 MHz) was recorded.

The chiral solvating reagent (S)-(+)-2,2,2-trifluoro-1-(9-anthryl) ethanol (7.0 mg, 25 μmol) was added, and the spectrum was rerecorded (see Figure 3.3).

δ	m	plus car	δ	m	comment
2.07	s		1.71	2s	baseline resolved, Δ = 2 Hz
3.25	m		2.86	2m	
3.70	d		3.34	2d	
4.20	dd		3.86	m	possibly 2dd
4.40	dd		3.98	ddd	
6.14	s		5.28	m	complex

When samples were determined to be non - racemic (ee > 10%), racemic material was added, and the spectrum was rerecorded.

5.3.2

Methyl Hydrogen (+)-*cis*-3-cyclobutene-1,2-dicarboylate (26)

Dimethyl *meso*-3-cyclobutene-1,2-dicarbonylate (24) (0.85 g, 5.0 mmol) was dissolved in phosphate buffer (pH 7.0, 67 mM, 10 ml) with stirring at 32°C. Pig liver esterase (260 U, free - prepared by spinning down 200 µl suspension, removing the supernatant and dissolving the pellet in 200 µl buffer, or immobilized on Eupergit C or Courtaulds immobilization polymer) was added. The decreasing pH was maintained at 7.00 by the addition of NaOH (0.5 M) from an automatic titrator. After the addition of 1.0 mol - equivalent of alkali (9 hours), the reaction stopped. The reaction mixture was acidified with HCl to pH < 2, and was then extracted with CH_2Cl_2 (4 x 50 ml). The organic extracts were combined, dried ($MgSO_4$) and the solvent was removed *in vacuo*, giving 0.75 g (96%) colourless oil 26. By the use of the chiral solvating reagent (S)-(-)-α-methylbenzylamine, the ee was determined to be 86%.

Yield = 96%.

$[\alpha]_D^{20}$ = +6.92° (c = 2.5, $CHCl_3$).

UV : λ_{max} (NaOH) 202 and 260 nm (ε 332 and 506).

IR (film) : ν 3,100 (m, hydrogen - bonded -OH), 1,730 (s, ester), 1,700 (s, unsaturated acid), 1,110 (m, C=O), 1,040 cm^{-1}

(C=O).

^{1}H NMR (220 MHz, $CDCl_3$ / TMS) : δ = 3.66 (s, 3H, CH_3O); 3.92 (s, 2H, $(CHCO_2)_2$); 6.25 (dd, 2H, J = 1.7 Hz, CH=CH).

^{13}C NMR (100.6 MHz, $CDCl_3$ / $CDCl_3$ = 77.0 ppm) : δ = 48.61 (=CHCHCO$_2$C); 48.86 (=CHCHCO$_2$N); 51.94 (CH_3OCO); 136.21 (CHCO$_2$C); 136.97 (CHCO$_2$N); 170.90 (CO_2CH_3); 176.03 (CO_2N).

MS (EI) : m/z = 156 (M^+), 139 (M^+ - OH), 125 (M^+ - OCH_3), 111 (M^+ - CO_2N).

High resolution MS : $C_7H_8O_4$, calc. 156.0422, found 156.0395.

5.3.3

Methyl (+)-*cis*-2-benzylcarbamoyl-3-cyclobutane carboxylate (35)

To a solution of (+)-26 (312 mg, 2.0 mmol) in anhydrous $CDCl_3$ (2.5 ml) was added 1,1'-carbonyldiimidazole (490 mg, 3.0 mmol). After the evolution of CO_2 had ceased (approximately 3 minutes), benzylamine (324 mg, 3.0 mmol) was added. ^{1}H NMR showed the ratio cis : trans to be 77 : 23 (by integration of the methyl singlets, δ = 3.60, 3.64). The solvent was removed *in vacuo*, and the crude product was purified by flash chromatography using ether (R_f = 0.2) as eluent. This gave 333 mg (68%) white crystals, mp = 107 - 108°C. These were recrystallised from CH_2Cl_2 - petrol, giving 270 mg white needles 35, mp = 108 - 108.5°C.

Yield = 55%.

mp = 108 - 108.5°C

Elemental analysis : Found : C, 68.5; H, 6.1; N, 5.7. $C_{14}H_{15}NO_3$ requires C, 68.5; H, 6.2; N; 5.7%.

$[\alpha]_D^{20}$ = +11.7° (c = 1.0, $CHCl_3$).

UV : λ_{max} (MeOH) 215, 254, 258, 262, 267 and 270 nm (ε 150, 25, 33, 39, 30 and 19).

IR (film) : ν 3,300 (s, amide), 3,080 (w, amide), 2,920 (w, alkane), 1,720 (s, ester), 1,645 (s, amide), 1,240 - 10 (m, doublet, C-O).

^1H NMR (400 MHz, $CDCl_3$ / TMS) : δ = 3.60 (s, 3H CH_3OCO); 3.92 (dt, 1H, J_{ab} = 5.0 Hz, J_{ax} < 0.5 Hz, $>CHCH_2$); 3.97 (dt, 1H, J_{ab} = 5.0 Hz, J_{bx} < 0.5 Hz, $>CHCH_2$); 4.43 (dq, 2H, J_{ab} = 14.3 Hz, J_{ax} = 5.8 Hz, $NHCH_2R$); 5.94 (br s, 1H, NH); 6.27 (dt, 1H, J_{ab} = 2.6 Hz, J_{ax} <0.5 Hz, -CH); 6.38 (dt, 1H, J_{ab} = 2.6 Hz, J_{bx} < 0.5 Hz, -CH); 7.27 (m, 5H, phenyl).

^{13}C NMR (100.6 MHz, $CDCl_3$ / $CDCl_3$ = 77.0 ppm) : δ = 43.67 (RCH_2N); 49.01 ($CHCONH$); 51.46 ($CHCO_2$); 51.84 (CH_3OCO); 127.44, 127.94, 128.58 (aromatic); 136.35 (=CH); 138.37 (=CH); 169.55 (CONH); 171.40 (CO_2).

MS (EI) : m/e = 245 (M^+), 186 (M^+ - CO_2CH_3), 160, 106.

High resolution MS : $C_{14}H_{15}NO_3$, calc. 245.1052, found 245.1062.

Attempted Synthesis of (+)-35

To a solution of 1,1'-carbonyldiimidazole (15 mg, 92 μmol) and benzylamine (11 mg, 102 μmol) in anhydrous $CDCl_3$ (0.6 ml), in a 5 mm NMR tube, was added (+)-26 (12 mg, 77 μmol). No CO_2 was evolved. The mixture was left stand overnight at room temperature. There was no evidence of amide formation at any stage by ^1H NMR. There was no evidence of the desired product by tlc. The reaction was not worked - up.

Cis - Trans Isomerization of the Imidazolamide intermediate (33)

To a solution of (+)-26 (90 mg, 0.58 mmol) in anhydrous $CDCl_3$ (0.6 ml), in a 5 mm NMR tube, was added 1,1'-carbonyldiimidazole (147 mg, 0.9 mmol). The isomerization of the imidazolamide intermediate was monitored by ^1H NMR at 90 MHz (by integration of the methyl singlets).

Table 5.1

Time (hours, mins)	*cis*	*trans*
0,00	100	0
0,10	40	60
0,25	14	86
0,40	11	89
1,00	11	89
5,00	11	89
25,00*	11	89

* additional 1,1'-carbonyldiimidazole was added.

Benzylamine (102 mg, 1.0 mmol) was added. The *cis* : *trans* ratio remained at 11:89.

Methyl (-)-*trans*-2-benzylcarbamoyl-3-cyclobutene carboxylate (34)

To a solution of (+)-(26) (136 mg, 0.87 mmol) in anhydrous $CDCl_3$ (1 ml) was added 1,1'-carbonyldiimidazole (230 mg, 1.42 mmol). There was a rapid evolution of CO_2 and the solution darkened. After one hour, ^1H NMR showed the cis : trans ratio of the imidazolamide intermediate to be 10 : 90 (by integration of the methyl singlets). Benzylamine (160 mg, 1.5 mmol) was added. The solvent was removed *in vacuo*, and the crude product was purified by flash chromatography using ether - petrol (2:1, R_f = 0.1) as eluant. This gave 181 mg (85%) white crystals 34, mp 71 - 73°C, $[\alpha]_D^{24}$ = - 196°. These were recrystallised from $CHCl_3$ - petrol, giving 139 mg (65%) white platelet crystals, mp 79 - 80°C.

Yield = 65%.

mp = 79 - 80°C

Elemental analysis : Found : C, 68.8; H, 6.0; N, 5.8. $C_{14}H_{15}NO_3$ requires C, 68.5; H, 6.2; N, 5.7%.

$[\alpha]_D^{20}$ = -253° (c = 0.88, $CHCl_3$).

UV : λ_{max} (MeOH) 229, 249, 254, 260, 265 and 269 nm (ε 180, 116, 139, 158, 144 and 118).

IR (film) : ν 3,270 (m, amide), 3,100 (w, amide), 2,950 (w, alkane); 1,740 (m, ester), 1,640 (s, amide), 1,230 (m, C-O).

^1H NMR (400 MHz, $CDCl_3$ / TMS) : δ = 3.66 (s, 3H, CH_3O_2C); 3.71 (d, 1H, J < 1 Hz, >CHC); 3.76 (d, 1H, J < 1 Hz,

>CHC); 4.38 (dq, 2H, J_{ab} = 11.9 Hz, J_{ax} = 5.7 Hz, $NHCH_2R$); 6.22 (dd, 2H, J_{ab} = 10.0 Hz, J_{ax} = 2.8 Hz, $(=CH)_2$); 6.27 (br s, 1H, NH); 7.27 (m, 5H, phenyl).

^{13}C NMR (100.6 MHz, $CDCl_3$ / $CDCl_3$ = 77.0 ppm) : δ = 43.44 (RCH_2N); 49.15 ($\underline{C}HCONH$); 50.50 ($\underline{C}HCO_2$); 51.85 ($\underline{C}H_3OCO$); 127.34, 127.55, 128.51 (aromatic); 137.33 (=CH); 137.42 (=CH); 170.21 (CONH); 171.70 (CO_2).

MS (EI) : m/z = 245 ($M^{\bullet}$), 186 ($M^{\bullet}$ - CO_2CH_3), 140, 106.

High resolution MS : $C_{14}H_{15}NO_3$: calc. 245.1052, found 245.1078.

5.3.4

Screening of Hydrolytic Enzymes for Selectivity Against *cis*-3-cyclobutene-1,2-dimethanol diacetate (25)

General Procedure

cis-3-cyclobutene-1,2-dimethanol diacetate (25) was dissolved in phosphate buffer (pH7.0, 67 mM, 4 ml) with stirring at 30 - 32°C. Enzyme (see table) was added. The decreasing pH was maintained at 7.00 by the addition of NaOH (0.1 M) from an automatic titrator. If there was a discernible rate change after the addition of one mol - equivalent of alkali, the reaction mixture was extracted with ethyl acetate (2 x 20 ml). The extract was dried ($MgSO_4$), and the solvent was removed *in vacuo*. The optical rotation of the sample was determined. If the value of $[\alpha]_D$ was found to be < 0.1°, then the sample was purified by column

chromatography using ethyl acetate as eluent. The ee was determined by the procedure outlined in section 5.5.1.

Table 5.2

Enzyme (Units)	Substrate (mmol)	Endpoint (equi.)	$[\alpha]_D$	ee(%)
PPL (Steapsin)(1,100)	0.18	1.0	-	8
Wheatgerm lipase (200)	0.17	2.0	-	-
ANL (750)	0.2	1.24	-0.7	2.4
CCL (10,000)	0.15	2.0	-	-
LP 142/II (10 mg)*	0.28	1.1	+2.0	40
Lipozyme (100 mg)	0.18	2.0	-	-
PPL (585)	1.00	1.0	+4.5	86.5
RJL (775)	0.15	0.6	-0.8	21
PLE (65)	0.35	2.0	-	-
HEAcE (250)	0.06	2.0	-	-
PLA_2 (140)	0.18	0.88	0.0	-
α-chy (700)	0.08	0.0	-	-
trypsin (1,100)	0.08	0.0	-	-

* Supplied by H. Schneider, Wuppertal.

5.3.5

Hydrolysis of the Diacetate 25 by Different Grades of Purity of Pig Pancreatic Lipase

25 (28 mg, 0.28 mmol) was dissolved in phosphate buffer (pH 7.0, 67 mM, 4 ml) with stirring at 32°C. Pig pancreatic lipase (2,000 U,) was added. The slowly decreasing pH was maintained at 7.00 by the addition of NaOH (80 mM) from an automatic titrator. The hydrolyses were not worked up.

Lipase Source	Activity (U/mg solid)	rate (% hydrolysis/ hour)
Sigma type II	13	5.0
Boehringer	600	1.6
Sigma type VI-S	16,400	0.5

Pig Pancreatic Lipase Catalysed Hydrolysis of the Diacetate 25 in the Presence of a Salt

Diacetate 25 (199 mg, 1.0 mmol) was dissolved in phosphate buffer (pH 7.0, 67 mM, 5 ml) containing NaCl (0.4 M) with stirring at 32°C. Pig pancreatic lipase (Steapsin, 260 U) was added. The decreasing pH was maintained at 7.00 by the addition of NaOH (0.1 M) containing NaCl (0.4 M, to maintain a constant ionic strength) from an automatic titrator. After the addition of one mol - equivalent of alkali (43 hours), the reaction mixture was extracted with ethyl acetate (3 x 25 ml). The organic extracts were combined, dried ($MgSO_4$) and the solvent was removed *in vacuo*. This gave 110 mg (70%) of a mixture of diacetate (25), monoacetate (27) and diol (7, 8% and 8% respectively by ^1H NMR). The

monoacetate was purified by column chromatography on silica using ethyl acetate as eluent. This gave 80 mg (51%) pure 27, $[\alpha]_D^{20}$ = +1.26° (c = 2.0, $CHCl_3$). The ee was determined to be 16% by the chiral analysis method outlined in section 5.5.1.

5.3.6

(+)-*cis*-3-cyclobutene-1,2-dimethanol monoacetate (27)

Optimised Procedure

Diacetate 25 (495 mg, 2.5 mmol) was dissolved in phosphate buffer (pH 7.0, 67 mM, 10 ml) with stirring at 25°C. *Pseudomonas fluorescens* lipase (Amano lipase P, 10 mg, 300 U) was added. The decreasing pH was maintained at 7.00 by the addition of NaOH (0.50 M) from an automatic titrator. After the addition of 0.8 mol - equivalents of alkali (18.5 hours), the reaction mixture was extracted with CH_2Cl_2 (3 x 50 ml). The aqueous component was saturated with NaCl and re - extracted with CH_2Cl_2 (3 x 50 ml). The organic extracts were combined, dried ($MgSO_4$) and the solvent was removed *in vacuo*, giving 410 mg (99.8%) mixture of 27 and 25. These were separated by flash chromatography using CH_2Cl_2 - ether (4:1, R_f 0.6 (25), 0.25 (27)) as eluent. This gave 79 mg (16%) 25, which was recycled, and 303 mg (73%) 27. By the use of the chiral solvating reagent (S)-(+)-2,2,2-trifluoro -1-(9-anthryl) ethanol, the ee of 27 was determined to be >97%. The minor enantiomer was not observed until racemic material was added.

Yield = 75%.

Elemental analysis : Found : C, 61.3; H, 7.7. $C_8H_{12}O_3$ requires C, 61.5; H, 7.75%.

$[\alpha]_D^{20}$ = +6.2° (c = 2.0, $CHCl_3$).

UV : λ_{max} (MeOH) 206 and 264 nm (ε 303 and 93).

IR (film) : ν 3,450 (hydrogen bonding), 3,050 (w, C=CH), 2,900 (m, CH_2), 1,740 (s, ester), 1390-70 (m, doublet, ester), 1,240 (s, C-O), 740 cm^{-1} (m, C-H).

^{1}HNMR (400 MHz, CCl_4 / C_6D_6 9:1) : δ = 2.07 (s, 3H, CH_3OC); 3.25 (m, 2H, (>CH)$_2$); 3.70 (d, 2H, J = 7.0 Hz, $CHCH_2OH$); 4.20 (dd, 1H, J_{ab} = 11.5 Hz, J_{ax} = 9.0 Hz, $CHC(H)HOCO$); 4.40 (dd, 1H, J_{ab} = 11.5 Hz, J_{ax} = 6.0 Hz, $CHC(H)HOCO$); 6.14 (s, 2H, (=CH)$_2$).

^{13}C NMR (100.6 MHz, $CDCl_3$ / $CDCl_3$ = 77.0 ppm) : δ = 20.84 ($\underline{C}H_3CO_2$); 64.31 ($\underline{C}H_2OCO$); 67.91 (CH_2OH); 82.04 (>CH); 84.00 (>CH); 137.37 (=CH); 138.04 (=CH); 170.59 (CO_2).

MS (EI) : m/z = 96, 67, 43.

MS (CI, NH_3) : m/z = 157, 96, 79.

High resolution MS (EI) : C_6H_8O, calc. 96.0575, found 96.0580.

High resolution MS (CI) : $C_8H_{13}O_3$ (MH^+), calc. 157.0865, found 157.0868. $C_8H_{12}O_3$, calc. 156.0783, found 156.0760.

5.3.7

(-)-*cis*-2-[(4-Phenyl)benzoyloxymethyl]-3-cyclobutene-1-methanol acetate (38)

To a solution of (+)-27 (242 mg, 1.55 mmol) in anhydrous pyridine (2.5 ml) was added 4-phenylbenzoyl chloride (408 mg, 1.87 mmol) with stirring. The mixture was stirred at room temperature overnight, and a white precipitate formed. Pyridine was removed under reduced pressure. The residue was dissolved in ether, filtered and the solvent was removed *in vacuo*, giving an off - white solid. This was purified by flash chromatography using ether - petrol (1:2, R_f 0.3) as eluent, giving 38 (521 mg, 99%, >97% pure by ^{1}H NMR). The sample was crystallised from petrol giving 445 mg (85%, pure by ^{1}H NMR). Recrystallization from either petrol or ethanol - water gave identical mp's and optical rotations to the original crystals.

Yield = 99% (prior to recrystallisation).

= 85% (1 recrystallisation).

mp = 61 - 62°C.

Elemental analysis : Found : C, 74.7; H, 6.1. $C_{21}H_{20}O_4$ requires C, 75.0; H, 6.0%.

$[\alpha]_D^{20}$ = -11.1° (c = 1.0, $CHCl_3$).

UV : λ_{max} (MeOH) 204 and 272 nm (ε 33,100 and 30,500).

IR (film) : υ 3,050 (w, C=CH), 2,950 (w, alkyl); 1,745 (s, acetyl ester), 1,720 (s, biphenyl ester), 1,230 (s, C-H), 1,100 (m, C-O), 750 (s), 700 cm^{-1} (m, aromatic, mono - substituted).

^{1}H NMR (220 MHz, $CDCl_3$ / TMS) : δ = 2.03 (s, 3H, CH_3CO_2); 3.35 (m, 1H, >CH); 3.45 (m, 1H, >CH); 4.35 (dd, 2H,

J_{ab} = 7.0 Hz, J_{ax} = 1.5 Hz, $CHCH_2O$); 4.50 (dd, 2H, J_{ab} = 7.0 Hz, J_{ax} = 1.5 Hz, $CHCH_2O$); 6.19 (dd, 2H, J_{ab} =7.0 Hz, J_{ax} = 1.5 Hz, $(=CH)_2$); 7.45 (m, 3H, aromatic); 7.70 (t, 4H, J = 9.0 Hz, aromatic); 8.15 (d, 2H, J = 9.0 Hz, aromatic).

^{13}C NMR (100.6 MHz, $CDCl_3$ / $CDCl_3$ = 77.0 ppm) : δ = 20.80 (CH_3); 44.37 (CH_2OAc); 44.43 (CH_2OCOR); 63.82 (>CH); 64.17 (>CH); 126.94, 127.14, 128.02, 128.80, 129.99 (aromatic); 137.73 (=CH); 137.80 (=CH); 166.13 (CO_2R); 170.81 (CO_2CH_3).

MS (EI) : m/z = 336 (M^+), 276 (M^+ - acetate), 198.

High resolution MS : $C_{21}H_{20}O_4$, calc. 336.1362, found 336.1334.

(+)-*cis*-2-[(4-Toluenesulphonyl)oxymethyl]-3-cyclobutene-1-methanol acetate (39)

To a solution of (+)-27 (80 mg, 0.51 mmol) in anhydrous pyridine (1 ml) was added 4-toluenesulphonyl chloride (tosyl chloride, 200 mg, 1.05 mmol) with stirring. The reaction had reached 90% overnight at room temperature by ^1H NMR, and the pyridine was removed under reduced pressure. The residue was dissolved in ether, dried ($MgSO_4$), filtered and the solvent was removed *in vacuo*, giving crude (+)-39 (204 mg, 129%). This was purified by flash chromatography using ether - CH_2Cl_2 (1:1, R_f 0.7) as eluent. This gave a colourless oil (110 mg, 0.35 mmol), pure by ^1H NMR.

Yield = 69%.

$[\alpha]_D^{20}$ = +7.6° (c = 2.2, $CHCl_3$).

^1H NMR (220 MHz, $CDCl_3$ / TMS) : δ = 2.00 (s, 3H, CH_3CO); 2.46 (m, 3H, CH_3R); 3.27 (m, 2H, $(>CH)_2$); 4.12 (d, 2H, J = 5.6 Hz, CH_2O); 4.14 (d, 2H, J = 7.0 Hz, CH_2O); 6.05 (dd, 2H, J_{ab} = 13 Hz, J_{ax} = 2.8 Hz, $(=CH)_2$); 7.35 (d, 2H, J = 8.0 Hz, p - substituted aromatic); 7.79 (d, 2H, J = 8.0 Hz, p - substituted aromatic).

^{13}C NMR (100.6 MHz, $CDCl_3$ / $CDCl_3$ = 77.0 ppm) : δ = 20.7 ($\underline{C}H_3CO_2$); 21.5 (CH_3R); 44.1; 44.2; 63.1; 69.8; 127.8, 129.7 (aromatic, unsubstituted); 132.9 (aromatic, CH_3 substituted); 136.7 (=CH); 138.3 (=CH); 144.7 (aromatic, S-substituted); 170.6 (CO_2).

MS (EI) : m/z = 250 (M^+ - acetate), 155, 138 (M^+ - tosyl).

MS (CI, NH_3) : m/z = 311 (MH^+), 250, 155, 139.

<u>cis-4-[(9-Phenylxanthen-9-yl)oxymethyl]-3-cyclobutene-1-methanol acetate (40)</u>

To a solution of (+)-27 (100 mg, 0.64 mmol) in anhydrous pyridine (1 ml) was added 9-chloro-9-phenylxanthene (200 mg, 0.99 mmol) with stirring. The reaction reached 90% within 6 hours at room temperature (by ^1H NMR), and the pyridine was removed under reduced pressure. The mixture was separated by flash chromatography using $CHCl_3$ - petrol (60 - 80, 1:1, R_f 0.36) as eluent. This failed to remove the majority of the

9-chloro-9-phenylxanthene, as did aqueous washing.

^1H NMR (400 MHz, $CDCl_3$ / TMS) : δ = 2.00 (s, 3H, CH_3CO_2); 3.32 (m, 1H, >CH); 3.43 (m, 1H, >CH); 4.30 (m, 2H, CH_2OCO); 4.49 (m, 2H, CH_2OC); 6.15 (d, 1H, J = 2.9 Hz, =CH); 6.19 (d, 1H, J = 2.9 Hz, =CH); 7.38 (t, 2H, J = 7.3 Hz, aromatic); 7.46 (t, 3H, J = 7.5 Hz, aromatic); 7.63 (m, 6H, aromatic); 8.10 (d, 2H, J = 8.3 Hz, aromatic).

^{13}C NMR (100.6 MHz, $CDCl_3$ / $CDCl_3$ = 77.0 ppm) : δ = 20.80 ($\underline{C}H_3CO$); 44.47 (CH_2); 44.52 (CH_2); 63.86 (>CH); 64.21 (>CH); 96.97 ($O\underline{C}(Ph)(C)_2$); 126.98, 127.17, 128.05, 128.83, 130.03 (aromatic); 137.78 (=CH); 137.85 (=CH); 139.93 ($(\underline{C}\text{-}C)_2O$); 145.65 ($(C)_2O$); 170.79 ($CO_2$).

5.4 Appendix

The objective behind the preparation of optically pure **27** was to provide chiral cyclobutene derivatives for electrocyclic ring opening and isomerisation processes (see section 5.1.1). To this end, work is currently in progress by T. Wallace and coworkers linking (+)-**27** with their synthetic programme. This can be achieved via the reaction scheme outlined below (T. Wallace, personal communications);

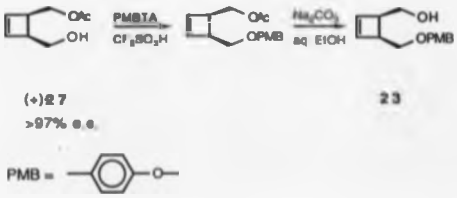


Figure 5.13 - Appendix scheme

The yields for the two stages are presently 46% and 72%. The procedure has not been optimised. The optical purity of the mono - PMB ether, **23**, can be determined via derivatisation with (R)-O-acetylmandelic acid using the method of Whitesell and Reynolds [128]. The ^1H NMR spectrum of the crude acetylmandelate is somewhat cluttered, making the accurate determination of the diastereomeric excess awkward. However, it is estimated to be between 70 - 90%.

Chapter 6 - Some Physical Studies of Steapsin

6.1 Introduction

Steapsin (Sigma type II lipase) is the trade name for a crude preparation of pig pancreatic lipase (triacylglycerol acylhydrolase, E.C. 3.1.1.3, PPL, also known as Pancreatin). During the course of this work, considerable variability was found between different batches of crude PPL preparations from the same supplier, and also especially between suppliers. This variability affected many parameters, including lipase activity, stereoselectivity and cosolvent characteristics. It should be recognised that such problems tend to reinforce the views of sceptics concerning the "black box" nature of biotransformations.

The variability arises from the crude nature of the preparations, including the many differing enzyme activities present. Typically, one of three approaches has been used when attempting to overcome these problems;

a) Purified preparations have been used.

b) The lipase activity has been enhanced by careful manipulation of the reaction conditions.

c) The unwanted activities have been reduced *in situ*.

Approaches b and c are not mutually exclusive. Pure preparations of PPL are commercially available. However, as discussed in section

1.4.4, it has been shown that the lipase in these preparations is probably not the desired chiral catalyst [62,64]. It is possible to inhibit competing enzymes by the use of specific inhibitors. This has been achieved with serine proteases [63]. Since steapsin contains such a diversity of activities, this approach should not be regarded as a universal panacea. Careful manipulation of the reaction conditions so as to maximise total lipase activity relative to other activities is probably the best technique for improving reproducibility with steapsin in the short term.

Because of the inconsistencies encountered with steapsin, all the work in this chapter was carried out with a single batch of Sigma type II lipase.

6.2 Results and Discussion

6.2.1 Determination of the Major Activities Present

The Api - zym (API Laboratory Products Ltd., Basingstoke) chromogenic substrate strip system was used to determine the presence of nineteen different enzyme activities. The results of this exercise are summarised in Figure 6.1. This system is only semi - quantitative, results being expressed on a 0 - 5 scale. Because of this, the proportion of total activity data should be treated with caution.

The system has three related esterase - lipase assays. They all use 2-naphthyl esters as substrates. Steapsin possesses

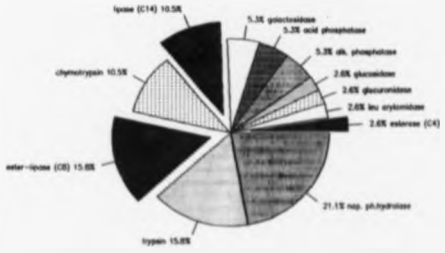


Figure 6.1 - Enzyme activities in steapsin

little esterase (butyrate, C_4) activity, but reasonable amounts of esterase/lipase (caprylate, C_8) and lipase (myristate, C_{14}) activity. In total, esterase - lipase activity forms just under one - third of the total activity of this sample of steapsin (using the 0 - 5 scale). Protease activity (chymotrypsin and trypsin) forms approximately one - quarter of the total. The greatest single activity appears to be phosphoamidase (naphthol phosphohydrolase), at one - fifth of the total.

It should be emphasized that the activities detected cannot necessarily be equated with individual proteins.

6.2.2 The Effect of Ionic Strength on Hydrolysis

The effects of high NaCl concentrations and of typical buffer concentrations on the rate of steapsin - catalysed hydrolysis of monomeric triacetin were investigated. As expected, increasing concentrations of NaCl produced linear increases in the hydrolysis rate up to 3 M NaCl (see Figure 6.2). Above this concentration the rate is effectively constant. NaCl reduces the aqueous solubility of triacetin, which then forms micelles. The correlation of the rate with salt concentration presumably reflects the dependence of the degree of micelle formation on the NaCl concentration, given that lipases display significantly higher activities towards aggregated substrates. It should be possible to alter both the position and gradient of the ionic strength v. activity curve by the use of different substrates, salts and initial substrate concentrations.

The enhancement of the hydrolysis rate produced by NaCl has previously been demonstrated using concentrated isotropic solutions of triacetin, tripropionin and 1,3-dibutyrin [129] and with emulsified triglycerides [130]. These systems required much lower NaCl concentrations to achieve maximum hydrolysis rates (typically 0.1 M), because the initial substrate concentrations were higher.

The effect of phosphate buffer concentration on the rate of steapsin - catalysed hydrolysis of monomeric triacetin was examined. The buffer salts concentration was varied between 0 - 67 mM; well below the critical micellar concentration for 50 mM triacetin. The results are summarised in Figure 6.3. Both the pre -

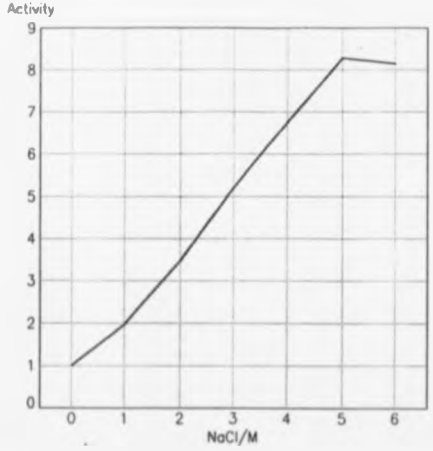


Figure 6.2 - The effect of NaCl on the rate of triacetin hydrolysis

steady state and steady state rates have been normalised. Both rates exhibit a maximum at 50 mM buffer. This coincidence of substrate and buffer concentrations is interesting. However, it would be necessary to vary both the substrate and its initial concentration before any conclusions could legitimately be drawn. Also worthy of comment is the absence of a pre - steady state phase in the absence of a buffer. Again, further experimentation would be required to establish the general validity of this observation.

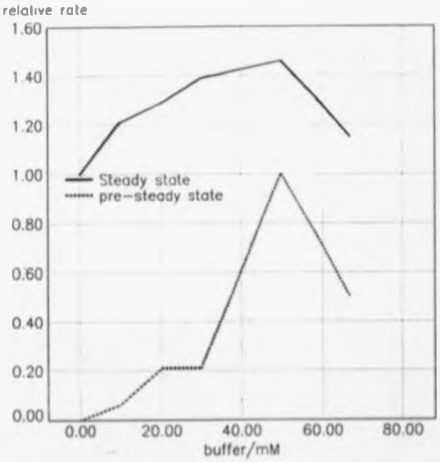


Figure 6.3 - The effect of buffer concentration on the rate of triacetin hydrolysis

6.2.3 Gel Filtration hplc

Three commercially available grades of PPL were examined by gel filtration hplc using a calibrated (see Figure 6.4) TSK G 3000 SW column.

Despite the high reproducibility obtained, little consistency was found between the samples. The most homogeneous

sample was the intermediate grade (600 U/mg, especially purified from proteases) from BCL. This gave two peaks. The higher molecular weight fraction was just over twice the mass of the smaller fraction, suggesting, given the limited accuracy of the technique, the possibility of monomer / dimer. The only fraction common between the two Sigma preparations (type VI-S, 10,400 U/mg and type II, 13 U/mg) was at 40 kd. This is significantly different to those of the BCL preparation (75 and 33 kd). The origin of this discrepancy is not clear. It is possible that, despite bearing the same name and E.C. number, the two sources have fractionated different proteins displaying lipase activity. PPL has been reported to have a mass of ca. 50 kd [131]. None of the preparations had a fraction within 5

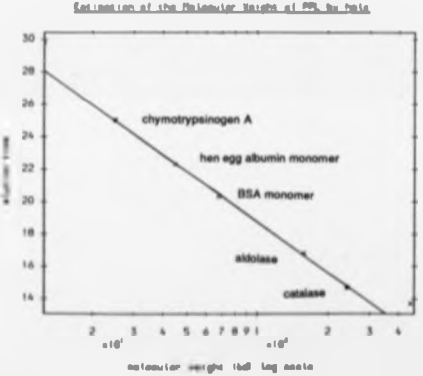


Figure 6.4

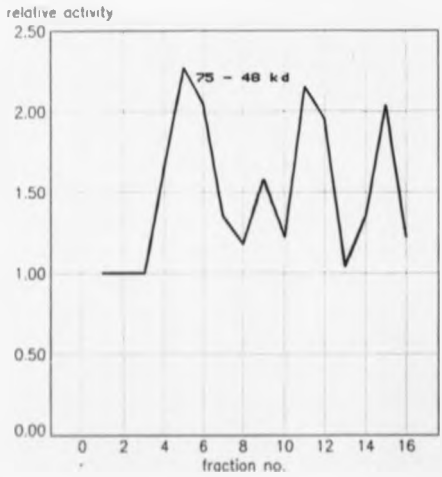


Figure 6.5 - Fractionation of crude PPL by hplc - activity profile

kd's of this value. The quality of the calibration curve is extremely good for the standard proteins with masses between 25 and 240 kd. However, it should be noted that some proteins do give anomalous molecular weights on TSK SW - type columns.

Attempts were made to fractionate steapsin using gel filtration hplc. These were hampered by the limited sample loading capacity of this type of column (<10 mg for an analytical column) and the low initial specific lipase activity of steapsin. This meant

that a maximum of 130 U could be chromatographed at any one time, and therefore large fractions had to be collected to provide sufficient activity for the lipase assay. Despite this, little differentiation between lipase activity and "noise" was obtained, even after subtraction of the background rate. The activity profile of a typical analysis is displayed in Figure 6.5. The large fraction volume led to broad molecular weight ranges for each fraction and hence to poor definition of the molecular weight of the lipase activity. The first activity peak covers the range 75 - 48 kd. This is the probable location of the lipase. The two other major activity peaks are likely to be non - enzymatic, as they correspond to mass ranges of 5 - 3 and <1 kd respectively.

6.3 Experimental for Chapter 6

6.3.1

Screening of Crude PPL (Steapsin) for Enzyme Activities

Crude PPL (Steapsin, 10 mg/ml) was assayed for 19 different enzyme activities using the semi - quantitative micromethod called API - ZYM (API Laboratory Products Ltd., Basingstoke, U.K.).

Crude PPL (65 µl per assay) was added to each of the 20 wells of the API - ZYM gallery, and H_2O (distilled, 5 ml) was added to the incubation tray. The gallery was incubated for 4 hours at 37°C. One drop of each of the two developing solutions (API ZYM A

and B) was added to each well. The colours were then allowed to develop under strong sunlight for 30 minutes. The results were recorded using the API - ZYM colour chart.

Gallery	Assay	Result (0 - 5)	nmoles	% total
1	control	0	-	-
2	alk. phosphatase	2	10	5.3
3	esterase (C_4)	1	5	2.6
4	esterase - lipase (C_8)	4	30	15.8
5	lipase (C_{14})	3	20	10.5
6	leucine arylamidase	1	5	2.6
7	valine arylamidase	0	-	-
8	cysteine arylamidase	0	-	-
9	trypsin	4	30	15.8
10	α-chymotrypsin	3	20	10.5
11	acid phosphatase	2	10	5.3
12	naphthol phosphohydrolase	5	>40	21.1
13	α-galactosidase	0	-	-
14	β-galactosidase	2	10	5.3
15	β-glucuronidase	1	5	2.6
16	α-glucosidase	0	-	-
17	β-glucosidase	1	5	2.6
18	N-acetyl-β-glucosaminidase	0	-	-
19	α-mannosidase	0	-	-
20	α-fucosidase	0	-	-

Total esterase - lipase activity 28.9 %.

Total protease activity 26.3 %.

Total phosphohydrolase activity 21.1 %.

6.3.2

The Effect of NaCl on PPL Activity

Lipase activity in the crude preparation of pig pancreas known as steapsin (or Pancreatin, 17 % protein, 13 lipase U/mg dry weight) was determined at a variety of ionic strengths.

The assay solution contained triacetin (triacetyl glycerol, 50 mM, from a stock solution), phosphate buffer (pH 7.0, 67 mM, from a stock solution), and NaCl (see below), to a total volume of 10 ml. This was equilibrated at 37°C with stirring. Pig pancreatic lipase (10 mg/ml, 200 µl) was added. The decreasing pH was maintained at 7.00 by the addition of NaOH (0.1 M) from an automatic titrator. The reaction was followed for 28 minutes.

[NaCl]/M	relative activity
0	1.00
1	1.97
2	3.47
3	5.20
4	6.75
5	8.28
6	8.14

The Effect of Phosphate Buffer Concentration on PPL Activity

Triacetin (50 mM, from a stock solution) and phosphate buffer (pH 7.0, from a stock solution) to a total volume of 10 ml, were equilibrated with stirring at 37°C. Pig pancreatic lipase (Steapsin, 10 mg/ml, 200 µl) was added. The decreasing pH was maintained at 7.00 by the addition of NaOH (0.1 M) from an automatic titrator. The reaction was followed for 28 minutes. Both the steady state and pre - steady state rates were determined.

The pre - steady state rates are expressed relative to the maximum pre - steady state rate observed. Steady state rates are expressed relative to that for no buffer.

[buffer]/mM	pre - steady state	steady state
0	0.00	1
10	0.04	1.21
20	0.21	1.29
30	0.21	1.39
50	1.00	1.46
67	0.50	1.15

6.3.3

Estimation of the Molecular Weight of PPL by Gel Filtration hplc

A TSK G 3000 SW gel filtration hplc column was calibrated with the following proteins; cytochrome c (12.5 kd), chymotrypsinogen A (25 kd), Hen egg albumin (45 kd), BSA (68 kd), aldolase (158 kd), catalase (240 kd) and ferritin (450 kd). The eluting solvent was phosphate buffer (pH 7.0, 67 mM), and the flow rate was 0.5 ml/min. Eluent was continuously monitored at 280 nm. The deadvolume was determined with dextran (254 nm).

Three different types of pig pancreatic lipase were examined;

1, Sigma type VI-S (16,400 U/mg).

2, BCL pig pancreatic lipase (600 U/mg).

3, steapsin (Sigma type II, 13 U/mg).

Sample	t_e (mins)	log mw (kd)
cytochrome c	24.8	1.097
chymotrypsinogen A	23.0	1.398
Hen egg albumin	22.3	1.653
BSA	20.3	1.833
aldolase	16.8	2.199
catalase	14.7	2.380
ferritin	13.7	2.653
dextran	13.2	>6

PPL Sample	t_a (mins)	Estimated mw (kd)
Sigma type VI-S	22.25	45.5
	22.9	39.5
	28.2	12.0
BCL	20.0	75.0
	23.8	33.0
Sigma type II	21.3	56.0
	22.8	40.0
	24.8	25.8
	26.0	19.8
	27.0	16.0
	29.2	10.0
	29.6	9.0
	31.25	6.0

Gel Filtration hplc of PPL and Fraction Activity

PPL (Steapsin, 5 mg/ml, 400 µl) was fractionated by gel filtration hplc using a TSK G 3000 SW column. The eluting solvent was phosphate buffer (pH 7.0, 67 mM), with a flow rate of 0.5 ml/min. The eluant was continuously monitored at 280 nm. Fractions (1 ml) were collected continuously from 6 minutes after injection. These were stored at 4°C.

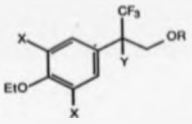
Each fraction was assayed against triacetin (0.3 M) in phosphate buffer (pH 7.0, 10 mM, 10 ml) containing NaCl (5 M) at 37°C for 20 minutes. Reaction rates were determined by the pH - stat method. Rates are expressed normalised against background.

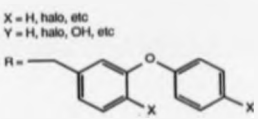
Fraction	relative rate	estimated mw (kd)
1	1.0	
2	1.0	
3	1.0	
4	1.67	116 - 75
5	2.27	75 - 48
6	2.04	48 - 31
7	1.35	31 - 20
8	1.18	20 - 16
9	1.58	16 - 8
10	1.22	8 - 5.2
11	2.15	5.2 - 3.3
12	1.95	3.3 - 2.1
13	1.04	2.1 - 1.4
14	1.35	1.4 - 0.9
15	2.04	0.9 - 0.6
16	1.22	

Chapter 7 - Method Development for Biotransformations - Resolution of an Insecticide Intermediate

7.1 Introduction

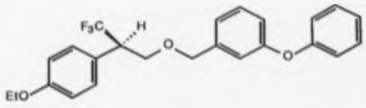
Until recently it was thought that pyrethroids required both a geminal dimethyl group and an ester moiety in order to possess insecticidal activity. However, two groups have now independently discovered a synthetic class of pyrethroids lacking the dimethyl group, but retaining insecticidal activity [132-137];





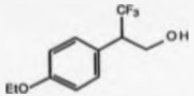
41

Molecules of this type have been shown to be active against a range of pests, including *Diabrotica balteata*, *Nilaparvata lugens* and *Tetranychus urticae*. One of the features retained throughout this class is a chiral centre adjacent to a trifluoromethyl group. It has been demonstrated that (*R*)-(+)-42 is 3.4 - 8 times as active as the corresponding (*S*)-(-) enantiomer, and up to twice as active as the racemate [137].



42

Previous resolutions of the chiral fragment have relied on fractional crystallisations of the corresponding acid with α-methylbenzylamine or on preparative chiral hplc methods [137]. Such procedures are tedious and impractical on a large scale. Clearly then, a procedure was needed for the resolution of the retained chiral fragment of 41 on a useful scale. Such a procedure was developed for 3,3,3-trifluoro-2-(4-ethoxyphenyl)propan-1-ol (43).



43

It was envisaged that the resolution would be achieved via an enantiospecific hydrolysis of an ester of 43. Additionally, the unwanted (S) enantiomer would be the preferred hydrolysis product. This would serve two purposes. Firstly, this would allow recycling of the unwanted enantiomer. The necessary racemisation of (S)-43 could be achieved by oxidation of the alcohol, followed by epimerisation and reduction. Secondly, the

enantiomeric excess of the residual (*R*) ester could be improved by carrying the hydrolysis beyond 50%, in the case of an incompletely stereospecific reaction. An e.e. of 80% was considered the minimum synthetically useful value for the residual ester. The ester could then easily be taken on to others such as 42.

This type of approach has proved useful for the resolution of 1-substituted-2,2,2-trifluoroethyl acetates with the lipase from *Candida cylindracea* (CCL) [138]. The *R* alcohol was the preferred product in this system, being produced with e.e.'s ranging between 55 - 98%. Other halo - substituted esters that have been subjected to hydrolytic biotransformation studies include methyl 2-chloropropionate [139] and various methyl 2-(chlorophenoxy)- propionates [140]. The former could be resolved by hydrolysis with CCL, but at the expense of the yield. The latter esters could be hydrolysed with moderate stereoselectivity (typically 60 - 70% e.e.) by chymotrypsin and CCL. Pig liver esterase and pancreatic lipase were not specific.

7.2 Results and Discussion

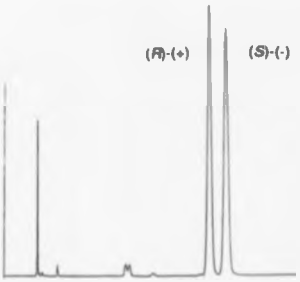
7.2.1 Chiral Analysis of 43

The racemic intermediate 3,3,3-trifluoro-2-(4-ethoxyphenyl)propan-1-ol (43), was supplied as a yellow oil by ICI Biological Products Division. The chiral solvating reagent (*S*)-(+)-2,2,2-trifluoro-1-(9-anthryl)ethanol (29) [118] was

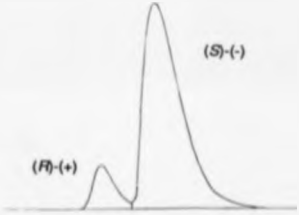
ineffective in the chiral analysis of the alcohol 43, either by ^{1}H or by ^{19}F (proton coupled and decoupled) NMR.

It has been reported that the enantiomers of 43 can be separated using a SUMIPAX OA-2000 chiral hplc column, using hexane / ethyl acetate (1000:1) as eluent [137]. Alcohol 43 can be separated on an ionic Pirkle type A1 column with hexane / ethanol as eluent (99:1, 1 ml/min) (M. Nicholds, personal communication). The particular type of column used consisted of 5 μm Spherisorb SSNH modified with D-3,5-dinitrobenzoyl phenylglycine (Hichrom Ltd., Reading). Isopropanol was used as the polar modifier in this work. Increasing the ratio of isopropanol reduced the elution times, but resulted in the incomplete separation of the enantiomers. Optimal separation was obtained with hexane / isopropanol (99:1) at a flow rate of 1.5 ml/min. This set-up gave near identical t_e values as the 1% ethanol system at 1 ml/min. A typical chromatogram is illustrated in Figure 7.1a. The required (R)-(+) enantiomer was the first enantiomer eluted (t_e = 22.1 mins). Since it was the alcohol that was being analysed, it was the second peak (t_e = 23.3 mins, (S)-(-) enantiomer) that was the preferred hydrolysis product.

Various types of charge - transfer, hydrogen - bonding, dipole stacking and steric effects are thought to be important in the separation mechanism of this type of column. Many of the compounds separated are hydrogen - bonding π - donors (e.g. heteroatom - substituted aromatics). The alcohol 43 clearly falls into this category.



a) (±)-**43**



b) PPL generated **43** (78% e.e.), from the hydrolysis of **44** in 50% isooctane (see section 7.2.7)

Figure 7.1 - Separation of **43** on a Pirkle type A1 chiral hplc column

7.2.2 Synthesis of Esters of 43

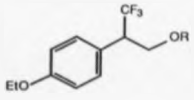
Six ester derivatives of 43 were prepared by treating the alcohol with the corresponding acid chloride in anhydrous pyridine. Acetate (44), propionate (45), butyrate (46), isobutyrate (47), valerate (48) and stearate (49) esters were synthesized (see Figure 7.2). Yields were typically 88 -91% after purification, except when some degree of polymerization occurred. The extent of the polymerization was greatly reduced (and often eliminated altogether) if 43 was purified (vacuum distillation or chromatography) prior to use, and if the reaction temperature was carefully controlled. When polymerization did occur, yields were typically 30 - 50%.

The purity of all of the esters was determined by hplc (see section 7.3.3) and was $\geq$97% in all cases. All of the esters except 49 were colourless oils. Ester 49 was crystalline. All esters were fully characterized.

7.2.3 Attempted NMR and hplc Resolution of Esters 44 - 49

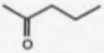
The ^{1}H and ^{19}F (proton coupled and decoupled) NMR spectra of 3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl acetate (44) were recorded in the presence of the chiral solvating reagent (S)-(+)-2,2,2-trifluoro-1-(9-anthyl)ethanol (29) [118]. No evidence was found for spectroscopic resolution of the enantiomers.

The esters 44 - 49 were analyzed by the chiral hplc



44 R =  acetate

45 R =  propionate

46 R =  butyrate

47 R =  isobutyrate

48 R =  valerate

49 R =  stearate

Figure 7.2 - Esters of **43**

method described in section 7.2.1. Although this system completely separates the enantiomers of the alcohol 43, it failed to produce any separation of the esters. All six esters were eluted in less than six minutes using 99:1 hexane / isopropanol. Separation of enantiomers by this type of column requires considerably longer residence times (typically 15 - 30 minutes). Comparison of these results with that for the alcohol 43 clearly demonstrates the importance of hydrogen - bonding for the separation mechanism.

7.2.4 Screening of Hydrolytic Enzymes for Selectivity Against 44

A comprehensive study of the enzyme - catalysed hydrolysis of 3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl acetate (44) was undertaken. A total of 42 different preparations, covering 33 hydrolytic enzymes from 5 suppliers were investigated. The hydrolysis reactions were monitored by the pH-stat technique at pH 7.0. The hydrolyses were allowed to proceed to 0.4 - 0.6 mol-equivalents or for 24 hours, whichever was shorter. The reactants and products were then extracted and analysed by the chiral hplc method outlined earlier. The results of this exercise are summarised in Tables 7.1 (lipases), 7.2 (proteases) and 7.3 (others). Since the extent of hydrolysis varied from one reaction to another, the "enantiomeric ratio" (E, see section 1.2.3) [16] is given. This allows the stereochemical efficacy of the different preparations to be directly compared.

None of the three *Candida cylindracea* lipase

preparations tested showed any degree of specificity towards ester 44. Given the utility of this lipase with other halogenated esters, especially 1-substituted-2,2,2-trifluoroethyl acetates [138], the results obtained were unexpected. Several other lipases produced promising E values. However, some of these were eliminated because of slow reactivity, problems of reproducibility or because they gave the wrong enantiomer. Given these caveats, the most interesting lipases were from pig pancreas (PPL), *Mucor javanicus* (MJL), *Rhizopus arrhizus* (RAL) and *R. delemar* (RDL). *Penicillium roquefortii* lipase was also promising, but it displayed the wrong specificity.

Three proteases gave interesting results. Thermolysin gave the desired enantiomer with moderate specificity, but the reaction was far too slow to be useful. Subtilisin displayed the wrong specificity. Newlase II was selected for further study.

Of the other hydrolases tested, only phospholipase A_2 was interesting. However, as with thermolysin, the reaction was extremely slow.

7.2.3 Newlase II Catalysed Hydrolysis of 44

Newlase II is a thermostable acid - protease from *Rhizopus sp.* prepared by Amano Pharmaceutical Co. (Frankfurt). It has optimal activity at pH 3. After the result at pH 7.0, it was thought that the enantiospecificity of the hydrolysis might be considerably improved if the reaction were carried out at a pH

nearer to the optimal value for this enzyme. This hypothesis was proved to not be correct. The enantioselectivity actually declined slightly as the pH was reduced. Because of this, no further work with Newlase II was undertaken.

7.2.6 Lipase Catalysed Hydrolysis of 44 in a Two - Phase System

The five lipases selected from the initial screening exercise (section 7.2.4) were analysed for stereoselectivity against ester 44 in a two - phase system. The water - immiscible solvent used was isooctane. The reaction system was sealed to prevent the gradual loss of isooctane through evaporation. Periodically, aliquots were withdrawn from the organic phase, and these were analysed by the chiral hplc method outlined previously. The results are summarised in Table 7.5.

The addition of a second phase brought about a greater than four - fold increase in the specificity of PPL, as expressed by the E value. The second phase made no difference to the RAL catalysed reaction, but reduced the enantiospecificity of the MJL, PRL and RDL catalysed hydrolyses. Given the interfacial characteristics of lipases, these reductions in specificity were unexpected. The E value obtained with PPL (6.8) suggests that the required 80% e.e. of the residual ester would be obtained at 62% hydrolysis. To obtain the ester optically pure would require the hydrolysis to be carried to 81%. Despite the slow rate of the PPL catalysed hydrolysis under the conditions employed, it was decided

that this type of system had the greatest potential as a resolution procedure for esters of 43.

7.2.7 Further Studies of the Steapsin Catalysed Hydrolysis of Esters of 43

A series of related experiments were instigated with the aim of optimising the stereospecificity of the Steapsin (PPL) catalysed hydrolysis of esters of 43 in a two - phase reaction system. Based on the four - fold increase in the E value obtained on the addition of a second phase, it was thought that further improvements could be obtained by additional manipulations of the reaction conditions.

The effect of the cosolvent on the enantiomeric ratio was investigated. Seven solvents were tested (see Table 7.6). They all produced significantly higher E values (6.2 - 12.7) than in the absence of a second phase (E = 1.65). With **44** as substrate, cyclohexane (E = 6.2) gave the smallest improvement. The best solvents were $CHCl_3$ (E = 12.7), ether (E = 10.6) and CCl_4 (E = 10.0) at 50% v/v, and isooctane (E = 10.0) at 75% v/v. With **48** as substrate, isooctane is a better cosolvent than $CHCl_3$. On the advice of ICI Biological Products Division, the use of halogenated solvents was avoided because of downstream processing problems. Ether was also avoided because of its high volatility and low flash point. (The reaction was performed at 37°C.) Isooctane had the best combination of the desired characteristics, and was used in

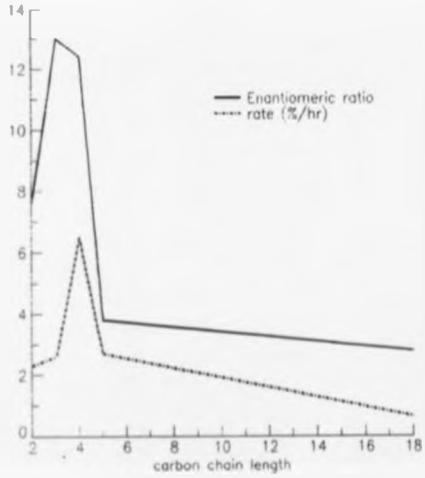


Figure 7.3 - The effect of the ester on PPL catalysed hydrolysis in a two - phase system

all subsequent experiments.

The effect of the ester chain length on the enantiomeric ratio was examined. All six derivatives prepared, 44 - 49, were hydrolysed with Steapsin in 50% isooctane. The results are summarised in Table 7.8 and graphically in Figure 7.3 (except the isobutyrate ester, 47). The type of ester has a significant effect on the enantiomeric ratio. The propionate (45) and butyrate (46)

esters are hydrolysed with nearly twice the selectivity (E = 12.4 - 13) of that for the acetate ester (44). The isobutyrate (47), valerate (48) and stearate (49) esters were hydrolysed with lower selectivity (E = 2.8 - 3.8). Although the selectivity for the propionate and butyrate esters is very similar, the hydrolysis rate is not. The butyrate ester (46) is hydrolysed at a rate that is nearly three times greater than those for the acetate, propionate and valerate esters. Clearly then, 46 combines both of the desired characteristics - high specificity and rapid hydrolysis. From the E value obtained for 46 (12.4), the required minimum 80% e.e. for the residual ester would be obtained at 54% hydrolysis under these reaction conditions. The ester would be optically pure at 69% hydrolysis.

It was found that the enantiomeric ratio was slightly higher at pH 7.0 than at pH 6.0 or 8.0. The results suggest that the optimum pH for specificity is probably pH 6.5 - 7.0.

The reaction system developed thus far involved only 0.25 mmol substrate. The effect of increasing the substrate concentration by a factor of four was ascertained with the acetate ester. This had the effect of increasing the E value from 7.6 to 10.6 (a factor of 1.4, see Figure 7.1b). This is probably an extension of the factors that came into play when the second phase was employed. That is, the substrate was removed from the domain of the non - interfacial and presumably non - specific enzymes. When dissolved in the organic phase, the substrate should only be bound and hydrolysed by lipases. Increasing the concentration of a sparingly water - soluble substrate in a two - phase system will

increase the proportion of the substrate dissolved in the organic phase. The fact that this procedure improved the selectivity of the reaction suggests that some proportion of the wrong enantiomer is being produced by non - selective hydrolysis in the aqueous phase. Further increasing the substrate concentration may produce additional improvements in the E value, as might reducing the substrate solubility in the aqueous phase (by, for example, having a high salt concentration - see section 6.2.2). An improvement of similar magnitude for the optimal substrate, 44, would give an E value of 17.4. In practice, this would mean that the minimum 80% e.e. for the residual (S)-(+) ester would be achieved at 51.4% hydrolysis, and optical purity at 63%. Undoubtedly, this procedure could be further improved.

The specific rotation of (S)-(-)-43 (resolved by fractional recrystallisation of the corresponding acid) has been reported as -29.8° [137]. A sample of the enzymatically produced alcohol (85% e.e., analysed by the chiral hplc method) had a rotation of -21.3°. Using the data of Tsushima *et al.*, this would suggest an e.e. of 71%. This is clearly an over - estimate. From the hplc data, the specific rotation should be of the order of 32.8°. The residual acetate had a rotation of +15.3°. The calculated e.e. for this sample (based on the extent of hydrolysis and the e.e. of the alcohol) was 86%. This suggests a specific rotation of 17.8° for 44. The sample was chemically hydrolysed, giving (S)-(+)-43. The chemically hydrolysed sample had a rotation of +30.8°, but was impure. It was difficult to determine the e.e. of this sample accurately by the chiral hplc method, because the major constituent

enantiomer was eluted first. Peak trailing by the major enantiomer partially obscured the minor enantiomer. However, the minimum e.e. was estimated to be >75%.

Experimental for Chapter 7

7.3.1

3,3,3-trifluoro-2-(4-ethoxyphenyl)propan-1-ol (43)

Yellow oil (90% by glc) as supplied by ICI. Pure colourless oil was isolated by flash chromatography with ether - petrol (1:2, R_f = 0.17) giving an 85% yield (98% pure by hplc, see section 7.3.2).

UV : λ_{max} (MeOH) 216, 274 and 280 nm (ε 11,200, 1,300 and 1,000).

^{1}H NMR (400 MHz, CCl_4 / C_6D_6) : δ = 1.30 (t, 3H, J = 7.0 Hz, CH_3CH_2O); 3.25 (m, 1H, $HCH(CF_3)CH_2$); 3.75 (dd, 1H, J_{ab} = 11.1 Hz, J_{ax} = 8.3 Hz, CHC(H)HOH); 3.82 (q, 2H, J = 7.0 Hz, CH_3CH_2O); 3.92 (dd, 1H, J_{ab} = 11.1 Hz, J_{bx} = 8.3 Hz, CHC(H)HOH); 6.70 (d, 2H, J = 8.6 Hz, p- substituted aromatic); 7.04 ppm (d, 2H, J = 8.6 Hz, p- substituted aromatic).

^{19}F NMR (84.67 MHz, $CHCl_3$ / $CFCl_3$) : δ = -68.42 ppm (d, J = 9.8 Hz).

Attempted Resolution of the Enantiomers of 43 by Proton NMR

3,3,3-trifluoro-2-(4-ethoxyphenyl)propan-1-ol (2.9 mg, 12.4 μmol) was dissolved in anhydrous CCl_4 / C_6D_6 (9:1, 0.5 ml) and it's ^1H NMR spectrum (400 MHz) was recorded.

(R)-(+)-2,2,2-trifluoro-1-(9-anthryl) ethanol (7.3 mg, 26.4 μmol, 2.1 mol equivalents) was added, and the spectrum was rerecorded. No peak splitting was observed.

Attempted Resolution of the Enantiomers of 43 by Fluorine NMR

3,3,3-trifluoro-2-(4-ethoxyphenyl)propan-1-ol (3 mg, 12.8 μmol) was dissolved in $CDCl_3$ (0.5 ml) and it's ^{19}F NMR spectrum (84.67 MHz, proton coupled and decoupled) were recorded.

(R)-(+)-2,2,2-trifluoro-1-(9-anthryl) ethanol (7.0 mg, 25.3 μmol, 1.98 mol equivalents) was added, and the spectra were rerecorded.

No peak splitting was observed for both the proton coupled and proton decoupled spectra.

Resolution of the Enantiomers of 43 by hplc

3,3,3-trifluoro-2-(4-ethoxyphenyl)propan-1-ol (43) was analysed by hplc using an ionic Pirkle type 1A column (Hichrom) consisting of 5 μm Spherisorb SSNH modified with D-3,5-dinitrobenzoyl phenylglycine. The eluting solvents were hexane - isopropanol (90:10 - 99:1) at 1.5 ml/min. Eluent was continuously monitored at 280 nm.

hexane :	isopropanol	R_t (mins)		Resolution*
		+	-	
90	10	5.61	5.61	0.33
95	5	8.11	8.45	0.63
97.5	2.5	12.72	13.33	0.70
99	1	22.08	23.33	0.93

* ratio of resolved peak height to total peak height

7.3.2

Synthesis of Esters of 43

3,3,3-trifluoro-2-(4-ethoxyphenyl)propan-1-ol (43) was added slowly to a stirred solution of acid chloride (1.5 mol equivalents) in anhydrous pyridine (1 ml/ mmol alcohol) under N_2 at 0°C, such that the reaction did not become violently exothermic. After 30 minutes, the reaction mixture was brought to room temperature, and stirred overnight. Pyridine was removed under high vacuum. The residue was taken up into ether, dried ($MgSO_4$), filtered and the solvent evaporated, to give the crude ester. The following esters were synthesised, all having been purified by flash chromatography.

Ester	Scale (mmol)	Yield (%)	Purity (%)*
acetate (44)	14.75	88	>99[1]
propionate (45)	4.2	41[2]	98
butyrate (46)	4.2	33[2]	>99[1]
isobutyrate (47)	3.85	90	>99[1]
valerate (48)	5.1	91	97
stearate (49)	3.7	89	98

* by hplc, using a Pirkle 1A - type column with hexane - isopropanol (99:1) as eluent. See section 7.3.3.

1 >99% means a single peak.

2 some polymerisation occurred.

3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl acetate (44)

Colourless oil isolated by flash chromatography with ether - petrol (1:5; R_f = 0.33), giving 3.47 g (88%).

bp: 100°C / 0.4 mm Hg.

Elemental analysis : Found : C, 56.1; H, 5.4. $C_{13}H_{15}F_3O_3$ requires C, 56.5; H, 5.5%.

UV : λ_{max} (MeOH) 216, 274 and 280 nm (ε 12,100, 1,700 and 1,500).

IR (film) : ν 3,000 (w, alkyl), 1,750 (s, ester), 1,640 (m, aromatic), 1,540 (m, aromatic), 1,250 (s, aromatic ether), 840 cm^{-1} (w, aromatic).

^{1}H NMR (400 MHz, $CDCl_3$ / TMS) : δ = 1.45 (t, 3H, J = 7.0Hz, OCH_2CH_3); 2.03 (s, 3H, CO_2CH_2); 3.25 (m, 1H, $RCH(CF_3)CH_2$); 4.10 (q, 2H, J = 7.0 Hz, OCH_2CH_3); 4.45 (dd, 1H, J_{ab} = 11.9 Hz, J_{ax} = 7.1 Hz, $CHC(H)HOC$); 4.55 (dd,

1H, J_{ab} = 11.5 Hz, J_{bx} = 7.1 Hz, CHC(H)HOC); 6.95 (d, 2H, J = 8.6 Hz, p - substituted aromatic); 7.30 (d, 2H, J = 8.6 Hz, p - substituted aromatic).

^{13}C NMR (100.6 MHz, $CDCl_3$ / $CDCl_3$ = 77.0 ppm) : δ = 14.65 ($\underline{C}H_3CH_2O$); 20.54 ($\underline{C}H_3CO_2$); 48.24 (q, J = 26.9 Hz, $\underline{C}F_3$); 61.94 ($CH_3\underline{C}H_2O$); 63.35 ($CHC\underline{H}_2OCO$); 114.64, 123.63, 124.37, 127.15, 129.90 (aromatic); 159.08 ($R\underline{C}H(CF_3)CH_2$); 170.40 ($\underline{C}O_2$).

^{19}F NMR (84.67 MHz, $CDCl_3$ / $CFCl_3$) : δ = -68.70 (d, J = 9.8 Hz).

MS (EI) : m/z = 276 (M^+), 216 (M^+ - acetate), 188 (216 - ether).

High resolution MS : $C_{13}H_{15}F_3O_3$, calc. 276.0974, found 276.0984.

3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl propionate (49)

Colourless oil isolated by flash chromatography with ether - petrol (1:5, R_f = 0.31), giving 0.50 g (41%).

Elemental analysis : Found : C, 57.8; H, 5.9. $C_{14}H_{17}F_3O_3$ requires C, 57.9; H, 5.9%.

UV : λ_{max} (MeOH) 216, 274 and 280 nm (ε 10,000, 1,500 and 1,300).

IR (film) : ν 3,000 (m, alkyl), 1,750 (s, ester), 1,640 (m, aromatic), 1,540 (s, aromatic), 1,250 (s, aromatic ether), 840 (w, aromatic), 735 cm^{-1} (m, alkane).

^1H NMR (400 MHz, $CDCl_3$ / TMS) : δ = 1.07 (t, 3H, J = 7.6 Hz, $\underline{C}H_3CH_2CO_2$); 1.40 (t, 3H, J = 7.0 Hz,

CH_3CH_2O); 2.27 (q, 2H, J = 7.6 Hz, $CH_3CH_2CO_2$); 3.65 (m, 1H, $RCH(CF_3)CH_2$); 4.01 (q, 2H, J = 7.0 Hz, CH_3CH_2O); 4.43 (dd, 1H, J_{ab} = 11.5 Hz, J_{ax} = 7.1 Hz, CHC(H)HOC); 4.51 (dd, 1H, J_{ab} = 11.5 Hz, J_{bx} = 7.1 Hz, CHC(H)HOC); 6.87 (d, 2H, J = 8.7 Hz, p - substituted aromatic); 7.19 (d, 2H, J = 8.7 Hz, p - substituted aromatic).

^{13}C NMR (100.6 MHz, $CDCl_3$ / $CDCl_3$ = 77.0 ppm) : δ = 8.80 ($CH_3CH_2CO_2$); 14.63 (CH_3CH_2O); 27.27 ($CH_3CH_2CO_2$); 48.30 (q, J = 26.6 Hz, CF_3); 61.80 (CH_3CH_2O); 63.37 ($CHCH_2OCO$); 114.67, 123.73, 124.41, 127.20, 129.92 (aromatic); 159.10 ($RCH(CF_3)CH_2$); 173.76 (CO_2).

MS (EI) : m/z = 290 (M^+), 216 (M^+ - propionate), 188 (216 - ether).

High resolution MS : $C_{14}H_{17}F_3O_3$, calc. 290.1130, found 290.1122.

3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl butyrate (46)

Colourless oil isolated by flash chromatography with ether - petrol (1:8, R_f = 0.32), giving 0.41 g (33%).

Elemental analysis : Found : C, 58.8; H, 6.3. $C_{15}H_{19}F_3O_3$ requires C, 59.2; H, 6.3%.

UV : λ_{max} (MeOH) 210, 274 and 280 nm (ε 11,500, 1,400 and 1,200).

IR (film) : ν 3,000 (m, alkyl), 1,730 (s, ester), 1,640 (m, aromatic), 1,540 (s, aromatic), 1,250 (s, aromatic ether), 840 (w, aromatic), 735 cm^{-1} (m, alkane).

^{1}H NMR (400 MHz, $CDCl_3$ / TMS) : δ = 0.87 (t, 3H, J = 7.4 Hz, $\underline{CH_3}CH_2CH_2$); 1.40 (t, 3H, J = 7.0 Hz, $\underline{CH_3}CH_2O$); 1.56 (sextet, 2H, J = 7.4 Hz, $CH_3\underline{CH_2}CH_2$); 2.23 (t, 2H, J = 7.4 Hz, $CH_2\underline{CH_2}CO_2$); 3.65 (m, 1H, $R\underline{CH}(CF_3)CH_2$); 4.01 (q, 2H, J = 7.0 Hz, $CH_3\underline{CH_2}O$); 4.44 (dd, 1H, J_{ab} = 11.5 Hz, J_{ax} = 7.2 Hz, $CHC(\underline{H})HOC$); 4.52 (dd, 1H, J_{ab} = 11.5 Hz, J_{bx} = 7.2 Hz, $CHC(H)\underline{H}OC$); 6.87 (d, 2H, J = 8.7 Hz, p - substituted aromatic); 7. 19 (d, 2H, J = 8.7 Hz, p - substituted aromatic).

^{13}C NMR (100.6 MHz, $CDCl_3$ / $CDCl_3$ = 77.0 ppm) : δ = 13.38 ($\underline{CH_3}CH_2CH_2$); 14.64 ($\underline{CH_3}CH_2O$); 18.14 ($CH_3\underline{CH_2}CH_2$); 35.85 ($CH_2\underline{CH_2}CO_2$); 48.27 (q, J = 27.1 Hz, CF_3); 61.71 ($CH_3\underline{CH_2}O$); 63.39 ($CHC\underline{H_2}OCO$); 114.70, 123.77, 124.42, 127.20, 129.94 (aromatic); 159.11 ($R\underline{CH}(CF_3)CH_2$); 172.97 ($CO_2$).

MS (EI) : m/z = 304 (M^+), 216 (M^+ - butyrate), 188 (216 - ether).

High resolution MS : $C_{15}H_{19}F_3O_3$, calc. 304.1286, found 304.1319.

3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl isobutyrate (47)

Colourless oil isolated by flash chromatography with ether - petrol (1:10, R_f = 0.36), giving 1.05 g (90%).

Elemental analysis : Found : C, 59.0; H, 6.7. $C_{15}H_{19}F_3O_3$ requires C, 59.2; H, 6.3%.

UV : λ_{max} (MeOH) 216, 274 and 280 nm (ε 13,000, 1,700 and 1,500).

IR (film) : ν 3,000 (m, alkyl), 1,750 (s, ester), 1,640 (m, aromatic), 1,540 (m, aromatic), 1,250 (m, aromatic ether), 840 cm^{-1} (aromatic).

^1H NMR (400 MHz, $CDCl_3$ / TMS) : δ = 1.06 (dd, 6H , J = 7.0, 5.1 Hz, $CH(CH_3)_2$); 1.40 (t, 3H, J = 7.0 Hz, CH_3CH_2); 2.48 (septet, 1H, J = 7.0 Hz, $CH(CH_3)_2$); 3.66 (m, 1H, $BCH(CF_3)CH_2$); 4.01 (q, 2H, J = 7.0 Hz, CH_3CH_2); 4.44 (dd, 1H, J_{ab} = 11.5 Hz, J_{ax} = 7.2 Hz, $CHC(H)HOC$); 4.48 (dd, 1H, J_{ab} = 11.5 Hz, J_{bx} = 7.2 Hz, $CHC(H)HOC$); 6.87 (d, 2H, J = 8.7 Hz, p - substituted aromatic); 7.20 (d, 2H, J = 8.7 Hz, p - substituted aromatic).

^{13}C NMR (100.6 MHz, $CDCl_3$ / $CDCl_3$ = 77.0 ppm) : δ = 14.60 ($\underline{C}H_3CH_2$); 18.60 ($CH(\underline{C}H_3)_2$); 33.74 ($\underline{C}H(CH_3)_2$); 48.27 (q, J = 27.2 Hz, CF_3); 61.67 ($CH_3\underline{C}H_2$); 63.36 ($\underline{C}HCH_2OCO$); 114.66, 123.73, 124.42, 127.21, 129.95 (aromatic); 150.09 ($B\underline{C}H(CF_3)CH_2$); 176.35 ($CO_2$).

MS (EI) : m/z = 304 ($M^{\bullet}$), 216 ($M^{\bullet}$ - isobutyrate), 188 (216 - ether).

High resolution MS : $C_{15}H_{19}F_3O_3$, calc. 304.1286, found 304.1282.

3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl valerate (6B)

Colourless oil isolated by flash chromatography with ether - petrol (1:5, R_f = 0.42), giving 1.48 g (91%).

Elemental analysis : Found : C, 60.0; H, 6.7. $C_{16}H_{21}F_3O_3$ requires C, 60.4; H, 6.65%.

UV : λ_{max} (MeOH) 216, 274 and 280 nm (E 11,000, 1,600 and 1,300).

IR (film) : υ 3,000 (m, alkyl), 1,750 (s, ester), 1,640 (m, aromatic), 1,540 (m, aromatic), 1,250 (s, aromatic ether), 1,170 (s, C-F), 840 (w, aromatic), 735 cm^{-1} (m, alkane).

^1H NMR (400 MHz, $CDCl_3$ / TMS) : δ = 0.85 (t, 3H, J = 7.3 Hz, $CH_3CH_2CH_2$); 1.24 (m, 2H, $CH_3CH_2CH_2$); 1.40 (t, 3H, J = 7.0 Hz, CH_3CH_2O); 1.51 (m, 2H, $CH_3CH_2CH_2$); 2.24 (t, 2H, J = 7.3 Hz, $CO_2CH_2CH_2$); 3.65 (m, 1H, $HCH(CF_3)CH_2$); 4.01 (q, 2H, J = 7.0 Hz, CH_3CH_2O); 4.44 (dd, 1H, J_{ab} = 11.5 Hz, J_{ax} = 7.2 Hz, $CHC(H)HOCO$); 4.51 (dd, 1H, J_{ab} = 11.5 Hz, J_{bx} = 7.2 Hz, $CHC(H)HOCO$); 6.87 (d, 2H, J = 8.7 Hz, p - substituted aromatic); 7.20 (d, 2H, J = 8.7 Hz, p - substituted aromatic).

^{13}C NMR (100.6 MHz, $CDCl_3$ / $CDCl_3$ = 77.0 ppm) : δ = 13.49 ($CH_3CH_2CH_2$); 14.65 (CH_3CH_2O); 21.99 ($CH_3CH_2CH_2$); 26.70 ($CH_3CH_2CH_2$); 33.67 (CO_2CH_2); 48.27 (q, J = 27.1 Hz, CF_3); 61.69 (CH_3CH_2O); 63.34 ($CHCH_2OCO$); 114.61, 123.67, 124.38, 127.16, 129.93 (aromatic); 150.08 ($HC(CF_3)CH_2$); 173.18 (CO_2).

MS (EI) : m/z = 318 (M^+), 216 (M^+ - valerate), 188 (216 - ether).

High resolution MS : $C_{16}H_{21}F_3O_3$, calc. 318.1443, found 318.1434.

3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl stearate (49)

White crystalline solid isolated by flash chromatography with ether - petrol (1:39, R_f = 0.3), and recrystallized from petrol, giving 1.63 g (89%).

mp : 43 - 43.5 °C.

Elemental analysis : Found : C, 69.65; H, 9.55. $C_{29}H_{47}F_3O_3$ requires C, 69.6; H, 9.52.

UV : λ_{max}(MeOH) 216, 274 and 280 nm (ε 15,700, 2,100 and 1,900).

IR (film) : ν 2,950 (s,alkyl), 1,750 (s, ester), 1,640 (w, aromatic), 1,540 cm^{-1} (s, aromatic).

^{1}H NMR (400 MHz, $CDCl_3$ / TMS) : δ = 0.87 (t, 3H, J = 7.0 Hz, $CH_3CH_2(CH_2)_n$); 1.24 (br, 28H, $(CH_2)_{14}$); 1.60 (t, 3H, J = 7.0 Hz, CH_3CH_2O); 1.51 (br t, 2H, $CO_2CH_2CH_2$); 2.23 (t, 2H, J = 7.5 Hz, CO_2CH_2); 3.65 (m, 1H, $HCH(CF_3)CH_2$); 4.01 (q, 2H, J = 7.0 Hz, CH_3CH_2O); 4.44 (dd, 1H, J_{ab} = 11.5 Hz, J_{ax} = 7.2 Hz, CHC(H)HOCO); 4.51 (dd, 1H, J_{ab} = 11.5 Hz, J_{bx} = 7.2 Hz, CHC(H)HOCO); 6.87 (d, 2H, J = 8.7 Hz, p - substituted aromatic); 7.19 (d, 2H, J= 8.7 Hz, p - substituted aromatic).

^{13}C NMR (100.6 MHz, $CDCl_3$ / $CDCl_3$ = 77.0 ppm) : δ = 13.98 ($CH_3(CH_2)_n$); 14.66 (CH_3CH_2O); 22.57 ($CH_2CH_2(CH_2)_n$); 24.66, 28.90, 29.11, 29.25, 29.31, 29.50 ($(CH_2)_n$); 31.81 ($CO_2CH_2CH_2$); 33.97 (CO_2CH_2); 48.27 (q, J = 27.0 Hz, CF_3); 61.66 (CH_2CH_2O); 63.33 ($CHCH_2OCO$); 114.68, 123.68, 124.37, 127.16, 129.93 (aromatic); 159.06 ($HCH(CF_3)CH_2$); 173.31 (CO_2).

MS (EI) : m/z = 500 (M^+), 267 (stearate), 216 (M^+ - stearate), 188 (216 - ether).

High resolution MS : $C_{29}H_{47}F_3O_3$. calc. 500.3477, found 500.3509.

7.3.3

Attempted Resolution of the Enantiomers of 44 by Proton NMR

3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl acetate (44) (3.8 mg, 13.8 μmol) was dissolved in anhydrous CCl_4 / C_6D_6 (9:1, 0.5 ml) and it's ^1H NMR spectrum (400 MHz) was recorded.

(S)-(+)-2,2,2-trifluoro-1-(9-anthryl) ethanol (7.6 mg, 27.5 μmol, 2.0 mol equivalents) was added, and the spectrum was rerecorded. No peak splitting was observed.

Attempted Resolution of the Enantiomers of 44 by Fluorine NMR

3,3,3-trifluoro-2-(4-ethoxyphenyl) propyl acetate (44) (3.2 mg, 11.6 μmol) was dissolved in $CDCl_3$ (0.5 ml) and it's ^{19}F NMR spectra (84.67 MHz, proton coupled and decoupled) were recorded.

(S)-(+)-2,2,2-trifluoro-1-(9-anthryl) ethanol (7.2 mg, 26.0 μmol, 2.2 mol equivalents) was added, and the spectra were rerecorded.

No peak splitting was observed for both the proton coupled and proton decoupled spectra.

Attempted Resolution of the Enantiomers of Esters of 43 by hplc

Esters of 3,3,3-trifluoro-2-(4-ethoxyphenyl)propan-1-ol (43) were analysed by hplc using an ionic Pirkle type 1A column

(Hichrom) consisting of 5 μm Spherisorb S5NH modified with D-3,5-dinitrobenzoyl phenylglycine. The eluting solvent was hexane - isopropanol (99:1) at 1.5 ml/min. Eluent was continuously monitored at 280 nm.

Ester	R_t (mins)	Resolution
acetate (44)	6.00	0
propionate (45)	4.00	0
butyrate (46)	3.83	0
isobutyrate (47)	3.63	0
valerate (48)	3.63	0
stearate (49)	3.31	0

7.3.4

Screening of Hydrolytic Enzymes for Selectivity Against 44

General Procedure

3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl acetate (44) (70 mg, 0.25 mmol) was added to phosphate buffer (pH 7.0, 67 mM, 5ml) with stirring at 37°C. Enzyme (see tables below) was added. The decreasing pH was maintained at 7.00 by addition of NaOH (0.1 M) from an automatic titrator. After the consumption of 0.4 - 0.6 equivalents of alkali (less if this point had not been reached in 24 hours), the mixture was extracted with hexane (5 ml), giving quantitative recovery. The extract was analysed in duplicate by the chiral hplc method outlined in section 7.3.1.

Table 7.1 : Lipases

Enzyme (units)	Source[1]	Hydrolysis	ee(%)(sign)[2]	rate[3]	E[4]
PPL (1,300)	S	0.27	21(-)	0.5	1.65
Wheatgerm (115)	S	0.07	8(-)	0.3	1.18
AML (3,000)	A	0.65	20(+)	3.1	2.06
(30 mg)*	B	0.34	9(+)	1.6	1.22
ASL (50 mg)*	B	0.60	15(-)	15.0	1.64
CCL (40 mg)*	A	0.58	3(-)	19.0	1.10
(49 mg)*	B	0.55	3(+)	2.2	1.10
(11,350)	S	0.52	3(-)	2.7	1.09
CLL (53 mg)*	B	0.37	2(+)	1.5	1.05
GCL (53 mg)*	B	0.64	15(+)	3.4	1.70
Lipozyme (47 mg)*	M	0.54	10(+)	1.3	1.35
MJL (1000)	A	0.60	42(-)	8.6	4.67
(34 mg)*	B	0.59	26(-)	1.4	2.37
PCL (60 mg)*	B	0.56	21(-)	2.3	1.93
PRL (50 mg)*	B	0.71	26(+)	2.8	3.85
PPL (600)	A	0.33	6(-)	2.5	1.16
(31 mg)*	B	0.37	3(-)	1.4	1.08
RAL (50 mg)*	B	0.24	21(-)	1.1	1.63
RDL (52 mg)*	B	0.18	31(-)	15.0	2.03
RJL (7,300)	A	0.37	6(+)	1.9	1.16
(29 mg)*	B	0.12	7(-)	0.6	1.16
RML (4,100)	A	0.17	8(-)	0.9	1.19
(32 mg)*	B	0.13	17(-)	0.5	1.44

Table 7.2 : Proteases

Enzyme (units)	Source[1]	Hydrolysis	ee(%)(sign)[2]	rate[3]	E[4]
Cl-chy (1,400)	S	0.61	10(-)	0.9	1.40
Newlase II (780)	A	0.17	33(-)	0.7	2.12
Papain (175)	S	0.10	7(+)	0.5	1.16
Strep. sp.(90)	S	0.04	12(+)	0.2	1.28
Protease2A (1000)	A	0.20	18(-)	4.0	1.50
Protease S (420)	A	0.08	16(-)	0.4	1.40
Prozyme 6 (2,800)	A	0.56	19(+)	2.9	1.82
Subtilisin (6000)	A	0.32	27(+)	1.3	1.96
(310)	S	0.10	4(+)	0.4	1.09
Thermolysin (710)	S	0.05	44(-)	0.1	2.63
Trypsin (1,000)	BCL	0.04	7(-)	0.2	1.15

Table 7.3 : Other Hydrolyases

Enzyme (units)	Source[1]	Hydrolysis	ee(%)(sign)[2]	rate[3]	E[4]
PLE (195)	BCL	0.60	16(+)	2.6	1.70
OPE (14)	S	0.40	6(+)	1.7	1.17
PPCE (21)	S	0.32	0	1.3	1.00
EEAcE (300)	S	0.37	0	0.5	1.00
PLA_2 (300)	S	0.09	26(-)	0.2	1.75
(350)	BCL	0.07	6(+)	0.2	1.13
Bromelain (75)	S	0.48	2(-)	2.2	1.06
Hyaluronidase(6750)S		0.27	8(+)	1.2	1.31

Key

1 A = Amano, B = Biocatalysts, BCL = Boehringer, N = Novo, S = Sigma

2 Refers to the predominant enantiomer of the product alcohol.

3 % hydrolysis / hour, averaged.

4 See [16].

* Activity not known.

7.3.3

Newlase II Hydrolysis of 44 at Various pH's

3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl acetate (44) (70 mg, 0.25 mmol) was added to phosphate buffer (for pH see Table 7.4, 67 mM, 5 ml) with stirring at 37°C. Newlase II (acid protease from *Rhizopus sp.*, 52 mg, 780 U) was added. The decreasing pH was maintained at the original pH by the addition of NaOH (0.1 N) from an automatic titrator. After a given period of time, the mixture was extracted with hexane (5 ml), and the extract was analysed in duplicate by the chiral hplc method outlined in section 7.3.1.

Table 7.4

pH	Hydrolysis (%) ee (%)[1]		rate[2]	E[3]
7.0	19	33	0.7	2.12
5.5	12	8	0.13	1.19
4.5	6	22	0.14	1.59
4.2	3	27	0.13	1.73

1 Refers to product alcohol.

2 % hydrolysis / hour, averaged.

3 See [16].

7.3.6

Lipase Hydrolysis of 44 in a Two - Phase System

Lipase (see Table 7.5) was dissolved in phosphate buffer (pH 7.0, 1 M, 5 ml) with stirring at 37°C. 3,3,3-trifluoro-1-(4-ethoxyphenyl)propyl acetate (44) (70 mg, 0.25 mmol) was dissolved in isooctane (5 ml). The two phases were mixed with stirring at 37°C in a stoppered round - bottomed flask. Periodically, aliquots were withdrawn from the organic phase and analysed in duplicate by the chiral hplc method outlined in section 7.3.1.

Table 7.5

Lipase (Units)	time(days)	hydrolysis(%)	ee(%)[1]	E[2]
PPL (1,300)	1	2	66	5.1
	2	3	69.4	
	5	6	74	6.8
	7	8	74	6.8
MJL (500)	1	1	27	
	2	6	30	
	5	18	28	1.9
	7	25	28	1.9
PRL (50 mg)	1	1	42	
	2	3	42	
	5	3	36	2.2
	7	3	36	2.2
RAL (51 mg)	1	2	28	
	2	3	28	
	5	4	15	1.4
	7	8	22	1.6
RDL (51 mg)	1	3	19	1.5
	2	6	19	1.5
	5	41	9.5	1.3
	7	48	13	1.4

1 Refers to the product alcohol.

2 See [16].

7.3.7

Screening of Water - Immiscible Cosolvents for Use with PPL

Part 1

3,3,3-trifluoro-2-(4-ethoxyphenyl) propyl acetate (44) (70 mg, 0.25 mmol) was dissolved in organic solvent (see Table 7.6). This was added to phosphate buffer (pH 7.0, 67 mM) containing PPL (100 mg, 1,300 U) with stirring at 37°C, to give a total volume of 10 ml. The decreasing pH was maintained at 7.00 by the addition of NaOH (0.1 M) from an automatic titrator. Once 0.4 - 0.6 equivalents of alkali had been consumed, aliquots were taken from the organic phase. These were analysed in duplicate by the chiral hplc method outlined in section 7.3.1.

Table 7.6

Cosolvent	(%v/v)	E	rate(%/hr)	lag time(mins)
none		1.65	0.5	15
isooctane	(33)	8.5	4.0	45
	(50)	7.6	2.3	90
	(75)	10.0	1.3	60
hexane	(50)	8.3	4.0	45
cyclohexane	(50)	6.2	1.9	30
diethyl ether	(50)	10.6	3.6	15
diisopropyl ether	(50)	8.1	2.7	30
chloroform	(50)	12.7	3.4	20
carbon tetrachloride	(50)	10.0	1.9	30

Part 2

As for section A, except that the substrate used was 3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl valerate (48) (80 mg, 0.25 mmol) and the cosolvent was always 50% (v/v).

Table 7.7

Cosolvent	E	rate(E/hr)	lagtime(mins)
Isooctane	5.8	2.0	60
chloroform	3.2	4.8	60

The Effect of Ester Chain Length on PPL Hydrolysis in a Two - Phase System

Various esters of 43 (0.25 mmol) were dissolved in isooctane (5 ml). This was added to phosphate buffer (pH 7.0, 67 mM, 5 ml) containing PPL (100 mg, 1,300 U) with stirring at 37°C. The decreasing pH was maintained at 7.00 by the addition of NaOH (0.1 M) from an automatic titrator. After the addition of 0.4 - 0.6 equivalents of alkali, aliquots were taken from the organic phase and these were analysed in duplicate by the chiral hplc method outlined in section 7.3.1.

Table 7.8

Ester	C length	E	rate(E/hr)	lagtime(mins)
acetate (44)	2	7.6	2.3	90
propionate (45)	3	13.0	2.6	0
butyrate (46)	4	12.4	6.5	20
isobutyrate (47)	4	3.1	1.1	300
valerate (48)	5	3.8	2.7	30
stearate (49)	18	2.8	0.65	120

The Effect of pH on the PPL Hydrolysis in a Two - Phase System

3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl acetate (44) (70 mg, 0.25 mmol) was dissolved in isooctane (5 ml). This was added to phosphate buffer (see table 7.9, 67 mM, 5 ml) containing PPL (100 mg, 1,100 U) with stirring at 37°C. The decreasing pH was maintained at the original pH by the addition of NaOH (0.1 N) from an automatic titrator. After the addition of 0.4 - 0.6 equivalents of alkali, aliquots were taken from the organic phase. These were analysed in duplicate by the chiral hplc method outlined in section 7.3.1.

Table 7.9

pH	E	rate (E/hr)	lagtime (mins)
6.0	6.0	2.8	85
7.0	7.6	2.3	90
8.0	5.2	1.0	35

The Effect of Substrate Concentration on PPL Hydrolysis in a Two - Phase System

3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl acetate (44) was dissolved in isooctane (5 ml). This was added to phosphate (pH 7.0, 67 mM, 5 ml) containing PPL (100 mg, 1,300 U) with stirring at 37°C. The decreasing pH was maintained at 7.00 by the addition of NaOH (0.1 M) from an automatic titrator. Periodically, aliquots were taken from the organic phase and these were analysed in duplicate by the chiral hplc method outlined in section 7.3.1.

Substrate (mmol)	mM	E	rate(%/hr)	rate(μmol/hr)
0.25	25	7.6	2.3	5.75
1.00	100	10.6	1.0	10.0

Determination of the Enantiomeric Excess of Residual 44

3,3,3-trifluoro-2-(4-ethoxyphenyl)propyl acetate (44) and 3,3,3-trifluoro-2-(4-ethoxyphenyl) propan-1-ol (43) (generated by PPL hydrolysis of 44) were separated by flash chromatography using ether - petrol (4:1) as eluent. 43 was analysed by the chiral hplc outlined previously, and the optical rotations of both 43 and 44 were determined.

Purified 44 was hydrolysed using KOH (1 M) in anhydrous absolute ethanol overnight. The solvent was removed *in vacuo*. The residue was taken up into ether, filtered and the solvent was removed *in vacuo*. This gave near - quantitative recovery of the hydrolysed alcohol (43), which was analysed by the chiral hplc

method outlined previously.

PPL generated **43**

$[\alpha]_D^{24}$ = -21.3° (c = 1.0, $CHCl_3$)

ee = 71% (from [137])

ee = 65% (hplc)

Residual **44**

$[\alpha]_D^{24}$ = +15.3° (c = 1.0, $CHCl_3$)

43, from chemically hydrolysed **44**

$[\alpha]_D^{24}$ = +20.8° ($CHCl_3$, impure)

ee > 70% (from [137])

ee > 75% (from hplc)

ee = 86% (calculated from % hydrolysis and

ee of PPL hydrolysed alcohol)

References

1 Crout, D.H.G. and Christen, M., in Modern Synthetic Methods, vol. 5, 1 - 114, ed. R. Scheffold, Springer - Verlag, Berlin, 1989.

2 Enzymes in Organic Synthesis (Ciba Foundation Symposium 111), Pitman, London, 1985.

3 Enzymes as Catalysts in Organic Synthesis, ed. M.P. Schneider, D. Reidel, Dordrecht, 1986.

4 Biocatalysts in Organic Media, eds. C. Laane, J. Tramper and M.D. Lilly, Elsevier, Amsterdam, 1987.

5 Jones, J.B., in Asymmetric Synthesis, vol. 5, 309 - 344, ed. J.D. Morrison, Academic Press, New York, 1985.

6 Sih, C.J. and Chen, C.S., Angew. Chem. Int. Ed. Engl., 1984, 23, 570.

7 Simon, H., Bader, J., Gunther, H., Neumann, S. and Thanos, J., Angew. Chem. Int. Ed. Engl., 1985, 24, 539.

8 Whitesides, G.M. and Wong, C.H., Angew. Chem. Int. Ed. Engl., 1985, 24, 617.

9 Jones, J.B., Tetrahedron, 1986, 42, 3351.

10 Wipf, B., Kupfer, E., Bertazzi, R. and Leuenberger, H.G.W., Helv. Chim. Acta, 1983, 66, 485.

11 Chambers, V.E.H., Denny, W.A., Evans, J.M., Jones, E.R.H., Kasal, A., Meakins, G.D. and Pragnell, J., J. Chem. Soc., Chem. Commun., 1972, 1300.

12 Glauser, B.I., Erne-Zellweger, D. and Mayerl, C., Helv. Chim. Acta, 1987, 70, 587.

13 Warwick Biotransformation Abstracts (1987-88), 1-2, Dept. of Chemistry, University of Warwick.

14 Jakovac, I.J., Goodbrand, H.B., Lok, K.P. and Jones, J.B., J. Am. Chem. Soc., 1982, 104, 4659.

15 Mialin, R., Ph.D. Thesis 4169, 1968, ETH, Zurich.

16 Chen, C.-S., Fujimoto, Y., Girdaukas, G. and Sih, C.J., J. Am. Chem. Soc., 1982, 104, 7294.

17 Chen, C.-S., Wu, S.-H., Girdaukas, G. and Sih, C.J., J. Am. Chem. Soc., 1987, 109, 2812.

18 Semenov, A.N., Khmelnitski, Y.L., Berezin, I.V. and Martinek, K., Biocatalysis, 1988, 1, 3.

19 Langrand, G., Baratti, J., Buono, G. and Triantaphylides, C., Biocatalysis, 1988, 1, 231.

20 Mahr, P., Wenspo-Sarcevic, N. and Tamm, C., Helv. Chim. Acta, 1983, 66, 230.

21 Schneider, M., Engel, N., Nounicke, P., Naimemann, G. and Gerish, N., Angew. Chem. Int. Ed. Engl., 1984, 23, 67.

22 Gais, H.-J. and Lukas, K.L., Angew. Chem. Int. Ed. Engl., 1984, 23, 142.

23 Sabbioni, G., Shaa, M.L. and Jones, J.B., J. Chem. Soc., Chem. Commun., 1984, 230.

24 Sabbioni, G. and Jones, J.B., J. Org. Chem., 1987, 52, 4565.

25 Jones, J.B., Himba, H.H. and Hultin, P.G., Can. J. Chem., 1985, 63, 452.

26 Ohno, M., Kobayashi, S., Iimori, T., Wang, Y.F. and Izawa, T., J. Am. Chem. Soc., 1981, 103, 2405.

27 Wang, Y.F., Izawa, T., Kobayashi, S. and Ohno, M., J. Am. Chem. Soc., 1982, 104, 6465.

28 Bjorkling, F., Boutelje, J., Gatenbeck, S., Hult, K., Norin, T. and Szmulik, P., Tetrahedron, 1985, 41, 1347.

29 Mohr, P., Rosslein, L. and Tamm, C., Helv. Chim. Acta, 1987, 70, 142.

30 Ramaswamy, S., Hui, R.A.H.F. and Jones, J.B., J. Chem. Soc., Chem. Commun., 1986, 1545.

31 Sicsic, S., Ikhel, M. and Le Goffic, F., Tetrahedron Lett., 1987, 28, 1887.

32 Alcock, N.W., Crout, D.H.G., Henderson, C.M. and Thomas, S.E., J. Chem. Soc., Chem. Commun., 1988, 746.

33 Crout, D.H.G., Gaudet, V.S.B., Laumen, K. and Schneider, M.P., J. Chem. Soc., Chem. Commun., 1986, 808.

34 Guanti, G., Banfi, L., Narisano, E., Riva, R. and Thea, S., Tetrahedron Lett., 1986, 27, 4839.

35 Eberle, M., Egli, M. and Seebach, D., Helv. Chim. Acta, 1988, 71, 1.

36 Kobayashi, S., Kamiyama, K., Iimori, T. and Ohno, M., Tetrahedron Lett., 1984, 25, 2557.

37 Gais, H.-J., Lukas, K.L., Ball, W.A., Braun, S. and Linder, H.J., Liebigs Ann. Chem., 1986, 667.

38 Adachi, K., Kobayashi, S. and Ohno, M., Chimia, 1986, 40, 311.

39 Park, D. and Jones, W.P., Fed. Proc., Fed. Am. Soc. Exp. Biol., 1974, 33, 1477.

40 Hamilton, R.E., Campbell, N.D., de Jersey, J. and Zerner, B., Biochem. Biophys. Res. Commun., 1975, 63, 1146.

41 Heymann, E. and Junge, W., Eur. J. Biochem., 1979, 95, 509.

42 Farb, D. and Jenks, W.P., Arch. Biochem. Biophys., 1980, 203, 214.

43 Heymann, E., Junge, W. and Krisch, K., FEBS Letts., 1971, 12, 189.

44 Junge, W., Krisch, K. and Hollandt, H., Eur. J. Biochem., 1974, 43, 379.

45 Long, R.M., Satoh, H., Martin, R.M., Kimura, S., Gonzalez, F.J. and Pohl, L.R., Biochem. Biophys. Res. Commun., 1988, 156, 864.

46 Choudhury, R.R., J. Histochem. Cytochem., 1972, 20, 507.

47 Kingsbury, H. and Masters, C.J., Biochem. Biophys. Acta, 1970, 200, 58.

48 Lipolytic Enzymes, Brockerhoff, H. and Jensen, R.G., Academic Press, New York, 1974.

49 Lipases, eds. B. Borgstrom and H.L. Brockman, Elsevier, Amsterdam, 1984.

50 Tsujita, T. and Brockman, H.L., Biochemistry, 1987, 26, 8423.

51 Tsujita, T. and Brockman, H.L., Biochemistry, 1987, 26, 8430.

52 Brish, L.E.S. and Tramper, J., Biotechnol. Bioengin., 1983, 27, 1258.

53 Xie, S.-P., Suemune, H. and Sakai, K., J. Chem. Soc., Chem. Commun., 1987, 838.

54 Suemune, H., Misuhara, Y., Ahita, H., Oishi, T. and Sakai, K., Chem. Pharm. Bull., 1987, 35, 3112.

55 Xia, Z.-H., Nakamura, I., Suemune, H. and Sakai, K., J. Chem. Soc., Chem. Commun., 1988, 966.

56 Guibe-Jampel, E., Rousseau, G. and Saluan, J., J. Chem. Soc., Chem. Commun., 1987, 1080.

57 Saluan, J. and Karkour, B., Tetrahedron Lett., 1987, 28, 4669.

58 Bianchi, D., Cahri, W., Casti, P., Francalanci, F. and Ricci, M., J. Org. Chem., 1988, 53, 104.

59 Miyano, S., Kawahara, K., Inoue, Y. and Hashimoto, H., Chem. Lett., 1987, 355.

60 Kasal, W., Hultin, P.G. and Jones, J.B., J. Chem. Soc., Chem. Commun., 1985, 1563.

61 Lauman, K. and Schneider, M., Tetrahedron Lett., 1985, 26, 2073.

62 Naumerla, M. and Gais, H.-J., Tetrahedron Lett., 1987, 28, 3471.

63 Patel, D.V., Van Middlesworth, F., Donnubauer, J., Gannett, P. and Sih, C.J., J. Am. Chem. Soc., 1986, 108, 4603.

64 Tombo, G.M.R., Schar, H.-P., Busquets, X.F. and Ghisalba, O., Tetrahedron Lett., 1986, 27, 5707.

65 Barker, D.L. and Jencks, W.P, Fed. Proc., 1967, 432.

66 Levy, M. and Ochoa, P., Fed. Proc., 1967, 816.

67 Junge, W. and Heymann, E., Eur. J. Biochem., 1979, 95, 519.

68 Dudman, N.P.B. and Zerner, B., Methods Enzymol., 1975, 35(B), 190.

69 Ochoa, P. and Levy, M., Biochim. Biophys. Acta, 1970, 213, 450.

70 Groenaid, P. and Jencks, W.P., Biochem., 1971, 10, 1210.

71 Adler, A.J. and Kistiakowsky, G.B., J. Biol. Chem., 1961, 236, 324.

72 Adler, A.J. and Kistiakowsky, G.B., J. Am. Chem. Soc., 1962, 84, 695.

73 Barker, D.L. and Jencks, W.P., Biochem., 1969, 8, 3890.

74 Ainslie, G.R., Shill, J.P. and Neet, K.E., J. Biol. Chem., 1972, 247, 7088.

75 Neet, K.E. and Ainslie, G.R., Trends Biochem. Sci., 1976 (July), 145.

76 Ui, N., Anal. Biochem., 1979, 97, 65.

77 Himmel, M.E. and Squire, P.G., Int. J. Peptide Protein Res., 1981, 17, 365.

78 Morgan, B.J., Webb, E.C. and Zerner, B., Biochem. Biophys. Res. Commun., 1966, 23, 18.

79 Krisch, K., Biochim. Biophys. Acta, 1966, 122, 265.

80 Lavine, L., Baer, A. and Jencks, W.P., Arch. Biochem. Biophys., 1980, 203, 236.

81 Huggins, C. and Lapides, J., J. Biol. Chem., 1947, 170, 467.

82 Bradford, M.M., Anal. Biochem., 1976, 72, 248.

83 Ecchichea, D.J., Can. J. Biochem., 1969, 47, 799.

84 European Patent Application 84 300416.8.

85 Laumen, K., Reimerdes, E.H., Schneider, M. and Gerisch, H., Tetrahedron Lett., 1985, 26, 407.

86 Still, W.C., Kahn, M. and Mitra, A., J. Org. Chem., 1978, 43, 2923.

87 Lam, L.K.P., Brown, C.M., De Jesu, B., Lyu, L., Teeno, E.J. and Jones, J.B., J. Am. Chem. Soc., 1988, 110, 4409.

88 a) Xia, Z.-F., Suemune, H., Nakamura, I. and Sakai, K., Chem. Pharm. Bull., 1987, 35, 4454. b) Olah, G.A., Fung, A.P. and Meider, D., Synthesis, 1981, 280.

89 Bjorkling, F., Boutelje, J., Gatenbeck, S., Hult, K., Norin, T. and Szmulik, P., Bioorg. Chem., 1986, 14,176.

90 Guanti, G., Banfi, L., Narisano, E., Riva, R. and Thea, S., Tetrahedron Lett., 1986, 27, 4642.

91 Agosta, W.C., J. Am. Chem. Soc., 1962, 84, 110.

92 Agosta, W.C., J. Am. Chem. Soc., 1964, 86, 2638.

93 Lowe, G., J. Chem. Soc., Chem. Commun., 1965, 411.

94 Brewster, J.H., Topics Stereochem., 1967, 2, 1.

95 Bertrand, M., Gil, G. and Kumar, K., Nouveau J. Chim., 1980, 4, 61.

96 Ruch, E. and Schoenhofer, A., Theor. Chim. Acta, 1968, 10, 91.

97 Ruch, E. and Schoenhofer, A., Theor. Chim. Acta, 1970, 19, 225.

98 Ruch, E., Accounts Chem. Res., 1972, 5, 49.

99 Ruch, E., Angew. Chem., 1977, 89, 67.

100 Lafever, F., Martin, M.-L. and Chapman, M.-L., Compt. Rend. (C), 1972, 275, 1387.

101 Claesson, A., Olsson, L.-I., Sullivan, G.R. and Mosher, H.S., J. Am. Chem. Soc., 1975, 97, 2919.

102 Olsson, L.-I. and Claesson, A., Acta Chem. Scand., 1977, B31, 614.

103 Macomber, R.S., J. Am. Chem. Soc., 1977, 99, 3072.

104 Lang, R.W. and Hansen, H.-J., Helv. Chim. Acta, 1979, 62, 1025.

105 Lang, R.W. and Hansen, H.-J., Helv. Chim. Acta,

1979, 62, 1458.

106 Gil, G., Parre, R., Meou, A., Le Petit, J. and Triantaphylides, C., Tetrahedron Lett., 1987, 28, 1647.

107 Mieszcwica, F.M. and Bloch, K., Biochem. Biophys. Res. Commun., 1975, 65, 331.

108 a) Brynes, T.A. and Dolak, T.M., Org. Syn., 57, 62.

b) Jones, E.R.H., Mansfield, G.H. and Whiting, M.C., J. Chem. Soc., 1954, 3208.

109 Green, R.H. and Lambeth, P.F., Tetrahedron, 1983, 39, 1687.

110 Adams, J., Fitzsimmons, B.J., Girard, Y., Leblanc, Y., Evans, J.F. and Rokach, J., J. Am. Chem. Soc., 1985, 107, 464.

111 Ingham, S., Turner, R.W. and Wallace, T.W., J. Chem. Soc., Chem. Commun., 1985, 1664.

112 Woodward, R.B. and Hoffmann, R., Angew. Chem. Int. Ed. Engl., 1969, 8, 781.

113 Bloomfield, J.J. and Owsley, D.C., Org. Photochem. Synth., 1976, 2, 36.

114 Brauman, J.I. and Archie, W.C., J. Am. Chem. Soc., 1972, 94, 4262.

115 Trost, B.M. and McDougal, P.G., J. Org. Chem., 1984, 49, 458.

116 Schneider, M., Engel, N., Honicke, P., Heinemann, G. and Gerlach, H., Angew. Chem. Int. Ed. Engl., 1984, 23, 67.

117 Sabbioni, G. and Jones, J.B., J. Org. Chem., 1987, 52, 4365.

118 Pirkle, E.J. and Hoover, D.J., Topics Stereochem., 1982, 13, 263.

119 Luhse, E.-L., FRG Patent DE 3613313 A1.

120 Staab, H.A., Angew. Chem. Int. Ed. Engl., 1962, 1, 351.

121 Paul, R. and Anderson, G.W., J. Org. Chem., 1962, 27, 2044 and references therein.

122 Circular Dichroic Spectroscopy - Exciton Coupling in Organic Chemistry, N. Harada and K. Nakanishi, University Science Books, Mill Valley, Ca., 1983.

123 Winter, R.E.K., Tetrahedron Lett., 1965, 1207.

124 Garratt, P.J. and Zahler, R., J. Am. Chem. Soc., 1978, 100, 7753.

125 Ader, U., Breitgoff, D., Klein, P., Lauman, K. and Schneider, M.P., Tetrahedron Lett., 1984, 30, 1793.

126 Corey, E.J., Albonico, S.M., Koelliker, U., Schaaf, T.K., and Varma, R.K., J. Am. Chem. Soc., 1971, 93, 1491.

127 Chattopadhyaya, J.B. and Reese, C.B., J. Chem. Soc., Chem. Commun., 1978, 639.

128 Whitesell, J.K. and Reynolds, D., J. Org. Chem., 1983, 48, 3548.

129 Entressangles, B. and Desmuelle, P., Biochem. Biophys. Acta, 1968, 159, 285.

130 Benzonana, G. and Desmuelle, P., Biochem. Biophys. Acta, 1968, 164, 47.

131 Verger, R., Sarda, L. and Desmuelle, P., Biochem. Biophys. Acta, 1971, 242, 580.

132 Bushell, M.J. and Raphael, R.A., Eur. Pat. Appl. EP 279,531.

133 Bushell, M.J., Whittle, A.J. and Carr, R.A.E., Eur. Pat. Appl. EP 311,361.

134 Bushell, M.J. and Carr, R.A.E., U.K. Pat. Appl. GB 2,187,452.

135 Tsushima, K., Matsuo, N., Tanabe, Y., Yano, T. and Hirano, M., Eur. Pat. Appl. EP 240,978.

136 Bushell, M.J. and Carr, R.A.E., U.K. Pat. Appl. GB 2,199,825.

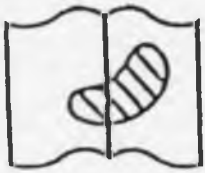
137 Tsushima, K., Yano, T., Takagaki, T., Matsuo, N., Hirano, M. and Ohno, N., Agric. Biol. Chem., 1988, 52, 1323.

138 Lin, J.T., Yamazaki, T. and Kitazume, T., J. Org. Chem., 1987, 52, 3211.

139 Dahod, S.K. and Siuta-Mangano, P., Biotechnol. Bioengin., 1987, 30, 995.

140 Dernoncour, B. and Amrad, R., Tetrahedron Lett., 1987, 28, 4661.

BEST COPY
AVAILABLE



TITLE Applications of Enzymatic Methods to the Preparation of Enantiomerically Pure Compounds

AUTHOR Ian Harvey

INSTITUTION and DATE University of Warwick 1989